Manipulation of Nitrogen Sink-source Relationship in Plants

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Abstract

Plant protein sources provide 65% of world supply of edible protein. However, most crop seeds are nutritionally incomplete due to insufficient synthesis and accumulation of several essential amino acids. Manipulation of nitrogen sink-source relationship to improve quantity and quality of seed proteins in crop seeds provides a cost-effective way to supply such amino acids. Metabolic pathways of aspartate family amino acids, which are all essential amino acids except asparagine, are major targets for seed quality improvement.

In this study, transgenic plants with altered sink-source relationship of aspartate family amino acid metabolism (35S-*ASN1*, phas-*PN2S*, phas-*LRP*, phas-*metL* and phas-*dapA*/phas-*LRP*) were analyzed. Increased methionine content was observed in mature seeds of transgenic plants expressing a methionine-rich protein and to a much lesser extent in transgenic plants expressing feedback insensitive AK-HSD. While expressing an effective sink for methionine enhanced the methionine content, expressing an effective sink for lysine did not improve the lysine content, and there is only slight increased in lysine level in transgenic plants expressing a combination of an effective sink for lysine and deregulated lysine synthesis.

In these transgenic plants, and also transgenic plants with enhanced source of asparagine from the source tissues, the total pool of aspartate family amino acids in the seeds did not increased. A down-regulation of expression of *akthr1* and *akthr2*, the two genes encoding AK-HSD which catalyzes the first committed step of aspartate family pathway, was observed in the transgenic plants with altered aspartate family amino acid metabolism during seed development and this maybe a factor hindering accumulation of aspartate family amino acids. The same down-regulation of gene expression of *AtGCN2* was observed in these transgenic plants. One the other hand, up-regulations of *akthr1* and *akthr2* were observed in transgenic plants overexpressing GCN2. These finding suggests that *akthr1* and *akthr2* are regulated by GCN2.

In this study, a multigene assembly vector system was employed to generate transgenic plants with a combination of manipulation of sink-source relationship of aspartate family amino acids. However, no transformant was obtained. Other systems for transformation of multiple genes simultaneously may be employed to generate the transgenic plants in future study.

摘要

植物蛋白來源佔全球食用蛋白供應的六成五。可是，大部分的種子蛋白由於數種必須氨基酸的合成和儲存不足而營養不全面。透過調控氮代謝的源庫關係來改良種子中蛋白質的質量與數量提供了一個合乎經濟效益的途徑供應這些氨基酸。因除天冬酰氨外，天冬氨酸家族氨基酸都屬於必須氨基酸，天冬氨酸家族氨基酸代謝途徑故此是改良種子質量的主要目標。

本研究對改變了天冬氨酸家族氨基酸代謝的源庫關係的轉基因植物(35S-*ASN1*, phas-*PN2S*, phas-*LRP*, phas-*metL* and phas-*dapA*/phas-*LRP*)進行了分析。在表達富含甲硫氨酸蛋白轉基因植物和表達反饋不敏感天冬胺酸激轉基因植物的成熟種子中，甲硫氨酸的含量提高了。提供有效甲硫氨酸貯存庫提高了甲硫氨酸的含量，但提供有效賴氨酸貯存庫沒有改善賴氨酸的含量，而在同時表達有效賴氨酸貯存庫和降低賴氨酸合成控制的轉基因植物中，賴氨酸的含量只有輕微上升。

在這些轉基因植物和增加了從源組織提供的天冬酰氨的轉基因植物中，天冬氨酸家族氨基酸的總含量沒有上升。*akthr1* 和 *akthr2* 基因轉譯成天冬胺酸激酶，催化天冬氨酸家族氨基酸合成途徑的第一個步，在改變了天冬氨酸家族氨基酸代謝的

轉基因植物中，*akthr1* 和 *akthr2* 在種子發育其間的基因表達降低了，這可能是阻礙天冬氨酸家族氨基酸儲存的一個因素。在這些轉基因植物中，*AtGCN2* 基因的表達出現同樣的基因表達下降。另一方面，在 *GCN2* 高表達轉基因植物中，*akthr1* 和 *akthr2* 基因的基因表達降低了。這些結果說明 *akthr1* 和 *akthr2* 是受 GCN2 的控制的。

本研究試驗利用一個多基因組合載體系統 (multigene assembly vector system) 去製造帶有一個組合的天冬氨酸家族氨基酸代謝的源庫關係調控的轉基因植物。可是，沒有得到成功的轉基因植物。未來研究可利用其他同時轉化多個基因的系統去製造這轉基因植物。

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Abbreviations

µl	Microlitre
µM	Micromolar
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
Asx	Aspartate and asparagine
bp	Base pair
CaMV	Cauliflower mosaic virus
Col-0	Columbia zero
Cys	Cysteine
DNA	Deoxyribonucleic acid
g	Gram
GAAC	General amino acid control
GCN	General Control Non-inducible
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
His	Histidine
Ile	Isoleucine
kb	Kilobase pair
Leu	Leucine
Lys	Lysine
M	Molar
Met	Methionine
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid

ng	Nanogram
PCR	Polymerase chain reaction
Phe	Phenylalanine
Pro	Proline
RNA	Ribonucleic acid
rpm	Revolution per minute
s	Second
Ser	Serine
T-DNA	Transferred DNA
Thr	Threonine
Ti-plasmid	Tumor-inducing plasmid
Trp	Trytophan
Tyr	Tyrosine
U	Unit
UV	Ultra-violet light
Val	Valine

Abbreviations of chemicials:

BSA	Bovine serum albumin
CI	Chloroform: isoamylalcohol
CTAB	Cetyldimethyethylammonium bromide
DEPC	Diethyl pyrocarbonate
dNTPs	Deoxyribonucleoside triphosphate
EDTA	Ethylenediamine-tetraacetic acid
EtOH	Ethanol
KCl	Potassium chloride
LB	Luria bertani broth, Miller
$MgCl_2$	Magnesium chloride
MgSO4	Magnesium chloride
MS	Murashige & Skoog salt mixture
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH_4OAc	Ammonium acetate
PCI	Phenol: Chloroform: isoamylalcohol
SDS	Sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
Tris base	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloric acid

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Chapter 1. Literature review

1.1 Significances of manipulation of nitrogen sink-source relationship

Plant protein sources contribute 65% of world supply of protein (Young and Pellett, 1994). Plant protein sources, with cereals predominant, are the major source of dietary protein for human and livestock, especially in developing countries (Millward, 1999).

Monogastric animals, such as human and poultry, cannot synthesize ten essential amino acids (cysteine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine) and need to obtain them in their diets (Lam *et al.*, 2006). However, most crop seeds, which are the major consumable parts, are nutritionally incomplete and are deficient in several essential amino acids. For examples, lysine (Lys) and methionine (Met) are two essential amino acids that are limiting in plant proteins and are required to be supplied by other means. While cereal seeds are deficient in Lys, legume seeds are deficient in Met (Galili and Hofgen, 2002).

Fortification of synthetic essential amino acids is a common solution to cope with amino acid deficiency of plant protein in developing countries. For example, a daily supplement of 2.4g Met to the abomasums of grazing sheep was shown to increase wool production by 22% (Pickering and Reis, 1993; Christiansen *et al.*, 2000).

However, this strategy is too costly for developing countries. It is estimated that increasing 1% Met content in soybean can save US$ 375 per year (Iowa State University and American Soybean Association, 1990). Moreover, doubling the Lys content in soybean can save US$ 3.6 per 100 pounds of soybean (Falco *et al.*, 1995). Thus, improvement of seed protein quantity and quality is of high agricultural and economical importance and has been the target of farmers and scientists for many years. Manipulation of nitrogen sink-source relationship to improve quantity and quality of seed proteins in crop seeds provides a cost-effective way to supply deficient amino acids.

1.2 Nitrogen sink-source relationship in plants

Plants obtain nitrogen in form of inorganic nitrogen, which must first be reduced to ammonium before assimilated into organic form for further metabolism (Crawford and Arst, 1993; Lam *et al.*, 1996). In non-legume plants, ammonium is absorbed from soil or is converted from nitrate by the concerted actions of nitrate reductase and nitrite reductase (Lea and Miflin, 1980; Miflin and Lea, 1980; Lea *et al.*, 1990). In legume plants, atmospheric nitrogen can be converted into ammonium in roots nodules by symbiotic *Rhizobium*. Nitrogen resources in form of nitrate and amino acids are transported from roots to leaves. Nitrogen resources will be transported as amino acids from source tissues to sink tissues, and the free amino acids transported to

seeds, the ultimate sink tissue, will be incorporated into seed storage proteins. To manipulate the seed protein quantity and quality, overall nitrogen sink source relationship in the whole plant (Figure 1.1) should be considered (Lam *et al.*, 2006).

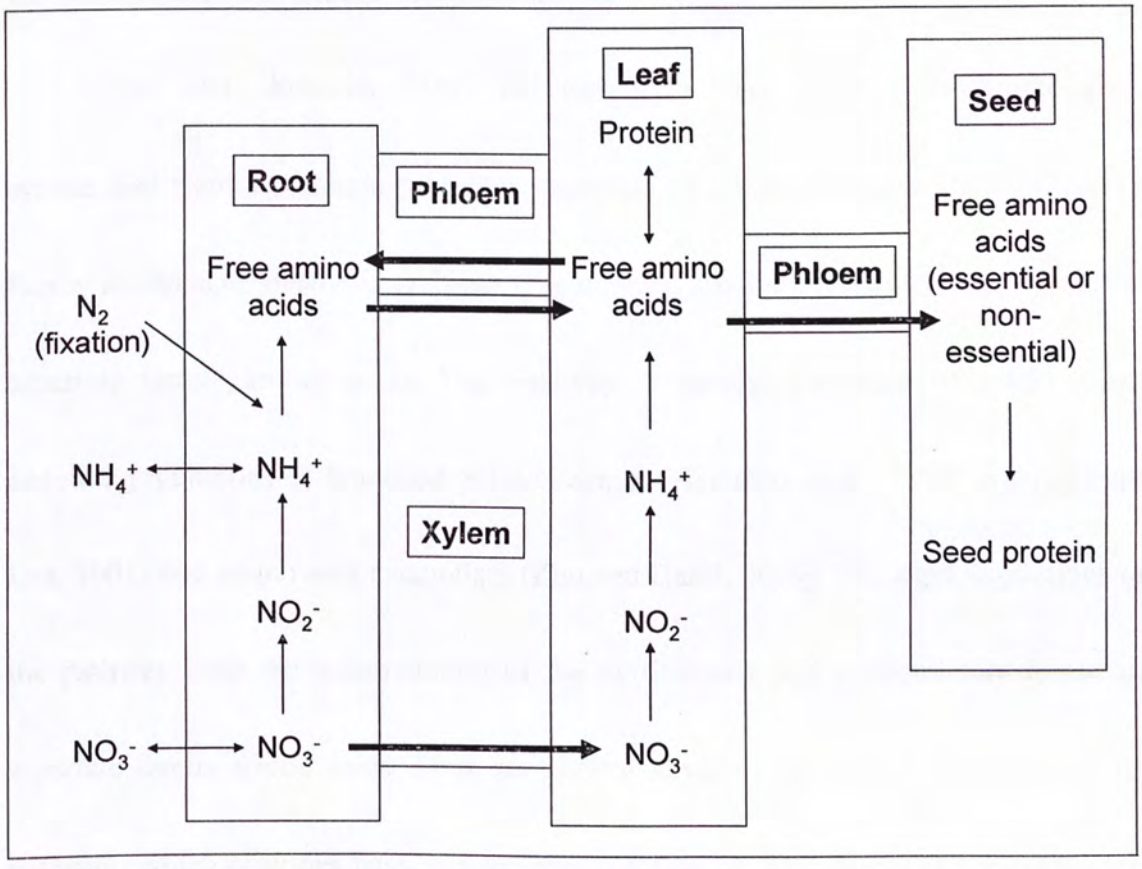


Figure 1.1 Overall nitrogen sink-source relationship leading to seed storage protein biosynthesis in plants.

Ammonium from soil or nitrate reduction or N fixation (in legumes) was assimilated into amino acids. Nitrogen resources will be transported as amino acids from source tissues to sink tissues, and the free amino acids transported to seeds will be incorporated into seed storage proteins.

1.3 Aspartate family amino acid metabolism

Lys, Met, threonine (Thr) and isoleucine (Ile) are essential amino acids synthesized from a common precursor, aspartate (Asp), through a branched aspartate family amino acid metabolic pathway (Figure 1.2), and they are collectively termed as aspartate family amino acids. The pathway is tightly controlled by positive and negative regulations of branched-point enzymes (Azevedo *et al.*, 1997; Azevedo and Lea, 2001) and amino acid catabolism (Zhu and Galili, 2004). The tight regulations on the pathway limit the accumulation of the nutritionally and economically important aspartate family amino acids. Thus, an understanding of the overall regulation of the pathway, which provides basis for the manipulation of these essential amino acids in seed proteins, is of great interest.

The common precursor of the aspartate family amino acid pathway, Asp, is also the substrate for another pathway leading to the synthesis of asparagine (Asn). As the two pathways compete for Asp as the substrate, Asn metabolism should also be considered when manipulating the aspartate family amino acids. While the Asn metabolism occurs at cytosol, nearly the entire aspartate family pathway is localized in chloroplasts or in plastid in non-photosynthetic tissues (Azevedo *et al.*, 2006; Lam *et al.*, 2006). The exceptions are the last step of Met synthesis and Met catabolism. Most of the previous researches suggested that methionine synthase (MS) is located in

cytosol, however, there was new evidence that an isoform of MS exists in chloroplast (Ravanel *et al.*, 2004).On the other hand, S-adenosylmethionine synthetase (SAMS) that catabolizes Met is located in cytosol (Ravanel *et al.*, 2004; Azevedo *et al.*, 2006) The key enzymes regulating the Asn and aspartate family amino acids metabolism are listed in Table 1.1 and discussed here.

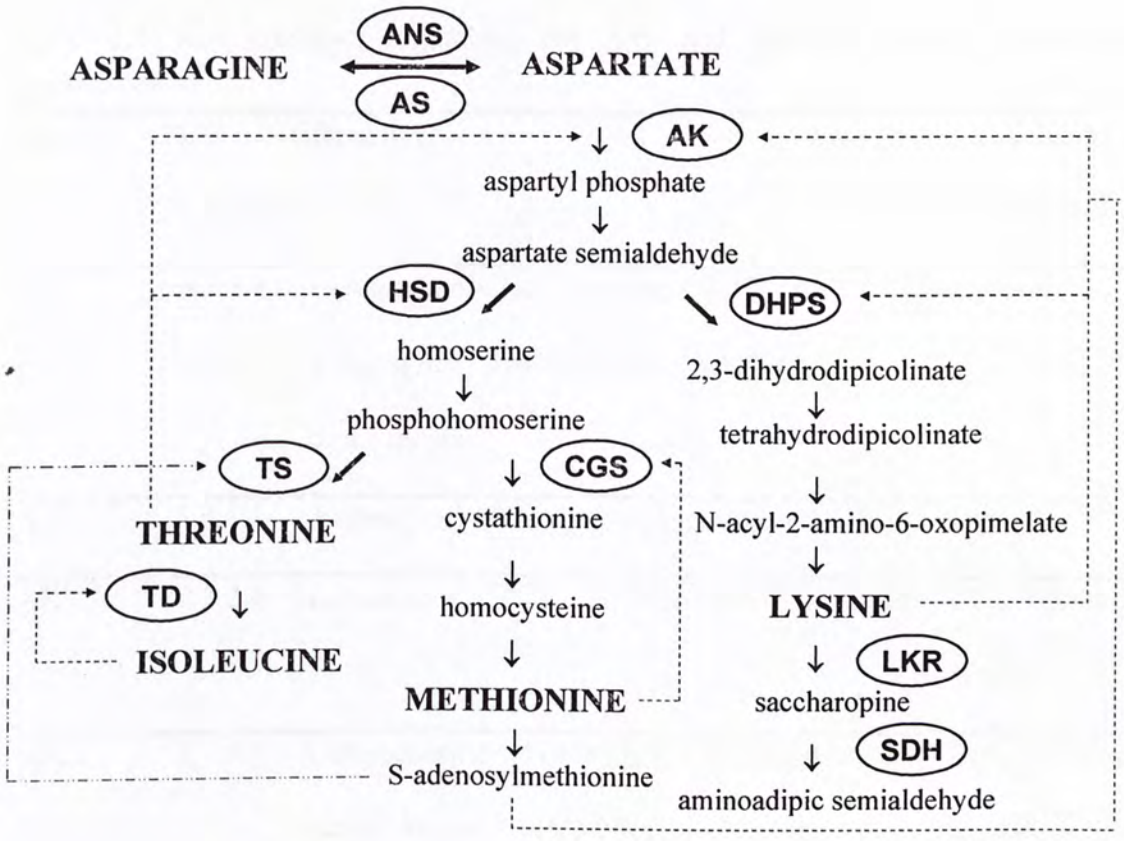


Figure 1.2 The metabolism of Asn and aspartate family amino acids. Major enzymes and allosteric regulations are shown. Solid arrows indicate metabolite flows; dash-arrows indicate allosteric feedback inhibitions; dash/dot-arrows indicate allosteric activations. AS, asparagine synthetase; ANS, asparaginase; AK, aspartate kinase; HSD, homoserine dehydrogenase; DHPS, dihydrodipicolinate synthase; CGS, cystathionine γ-synthase; TS, threonine synthase; TD, threonine dehydratase; LKR, lysine-ketoglutarate reductase; SDH, saccharopine dehydrogenase.

Table 1.1 Key enzymes regulating the Asn and aspartate family amino acid metabolism

Enzyme	**EC number**	**Reaction**	**Subcellular localization**
AS	6.3.5.4	L-Aspartate + L-Glutamine + ATP <=> L-Asparagine + L-Glutamate + AMP + diphosphate	Cytosol
ANS	3.5.1.1	Asparagine + H(2)O <=> Aspartate + NH(3)	Cytosol
AK	2.7.2.4	L-Aspartate + ATP <=> 4-phospho L-aspartate	Chloroplast and plastid
HSD	1.1.1.3	L-homoserine + NAD(P)(+) <=> L-aspartate 4-semialdehyde + NAD(P)H	Chloroplast and plastid
DHPS	4.2.1.52	L-aspartate 4-semialdehyde + pyruvate <=> dihydrodipicolinate + 2H(2)O	Chloroplast and plastid
LKR	1.5.1.7	L- Lysine + 2-oxoglutarate + NADH <=> N(6)-(L-1,3-dicarboxypropyl)-L-lysine + NAD(+) + H(2)O	Chloroplast and plastid
TS	4.2.3.1	o-phospho-L-homoserine + H(2)O <=> L-threonine + phosphate	Chloroplast and plastid
TD	4.2.1.16	L-threonine + NAD(+) <=> L-2-amino-3-oxobutanoate + NADH	Chloroplast and plastid
CGS	2.5.1.48	o-succinyl-L-homoserine + L-cysteine <=> cystathionine + succinate	Chloroplast and plastid

1.3.1 Asparagine metabolism

1.3.1.1 Asparagine synthetase (AS, EC 6.3.5.4)

Asn is an ideal form of transporting nitrogen due to its high N:C ratio and relative inertness (Lea and Miflin, 1980; Urquhart and Joy, 1981; Sieciechowicz *et al.*, 1988), and transports nitrogen between source and sink tissues (Lam *et al.*, 1995; Lam *et al.*, 1998). In certain legume species, Asn can account for up to 86% of the transporting nitrogen (Lea and Miflin, 1980).In higher plants, the major route of Asn production is via the glutamine-dependent AS. Glutamine-dependent AS catalyzes the synthesis of asparagine, by transferring the amide group from glutamine (Gln) to Asp to form glutamate (Glu) and Asn in an ATP-dependent reaction.

In many plant species, AS enzymes are encoded by a small gene family of one to three genes (Azevedo *et al.*, 2006). In *A. thaliana*, a small gene family, *ASN1*, *ASN2*, *ASN3*, encoding AS isoenzymes were identified previously (Lam *et al.*, 1994; Lam *et al.*, 1998).The biochemical studies of AS enzyme are hampered by the instability of the enzyme (Huber and Streeter, 1985), the presence of enzyme inhibitors (Streeter, 1973), the presence of asparaginase which breaks down Asn (Joy *et al.*, 1983), and the presence of isoenzymes which are the gene products of the *ASN* gene family (Lam *et al.*, 1998). Therefore, molecular genetics approaches are widely adopted to study the physiological roles of AS enzymes.

ASN1 and *ASN2* expressions were found to be reciprocally regulated by carbon and nitrogen metabolites while *ASN3* mRNA level is not detectable in leaf tissues (Lam *et al.*, 1998). Previous studies on the *ASN1* over-expressers demonstrated that *ASN1* over-expression can enhance the N transport and cause an increase in the seed protein content (Lam *et al.*, 2003).

1.3.1.2 Asparaginase (ANS, EC 3.5.1.1)

ANS catalyzes the conversion of asparagine to aspartate, providing the substrate for synthesis of aspartate family amino acids. There are two main families of ANS, the bacterial-type and the plant-type ANS. The plant-type ANS has both asparaginase activity and isoaspartyl peptidase activity, and can deaminate not only Asn but also isoaspartyl dipeptides (Hejazi *et al.*, 2002). On the other hand, the *E. coli* asparaginase II (encoded by *ansB*), which is a bacterial-type ANS, has no isoaspartyl peptidase activity and cannot hydrolyse isoaspartyl dipeptides. The *E.coli* asparaginase II has higher affinity towards Asn and hydrolyses Asn more readily than plant-type ANS (Hejazi *et al.*, 2002).

1.3.2 Metabolism of aspartate-derived essential amino acids

1.3.2.1 Aspartate kinase (AK, EC 2.7.2.4)

AK is the first enzyme committed to synthesis of all aspartate family amino acids. It catalyzes the phosphorylation of L-aspartate to form L-4-aspartyl phosphate. There are two classes of AK isoenzymes, the Lys-sensitive monofunctional AK enzyme and the Thr-sensitive bifunctional enzyme of AK and homoserine dehydrogenase (HSD) (Azevedo *et al.*, 2006). The monofunctional AK, which normally accounts for the majority of the AK activity, is feedback inhibited by lysine, and S-adenosylmethionine (SAM) can act synergistically with Lys to inhibit the AK activity (Rognes *et al.*, 1980). The existence of bifunctional AK has been reported in some but not all bacterial species (Cohen and saint-Girons, 1987). In *E. coli*, two bifunctional AK isoenzymes, AK-I/HSD-I and AKII/HSD-II, were found (Cohen and saint-Girons, 1987). The purification of a bifunctional protein with both AK and HSD activities from carrot suggested that bifunctional AK/HSD isoenzymes also exist in plants (Wilson *et al.*, 1991). The cloning of *AK/HSD* cDNA from carrots (Weisemann and Matthews, 1993) and the *AK/HSD* gene, designated *akthr1*, from *A. thaliana* (Ghislain *et al.*, 1994) further confirmed the existence of AK/HSD bifunctional isoenzymes in plants.

In *A. thaliana*, three genes that encode Lys-sensitive monofunctional AK, *AK-lys1* (Frankard *et al.*, 1997), *AK-lys2* (Tang *et al.*, 1997b) and *AK-lys3* (Yoshioka *et al.*, 2001), have been cloned. For bifunctional AK/HSD, besides *akthr1* (Ghislain *et al.*,

1994), a new cDNA, *akthr2*, was cloned by functional complementation of a *Saccharomyces cerevisiae* strain mutated in HSD gene (*hom6*) (Rognes *et al.*, 2003). Expression pattern of *akthr1* and *akthr2* were studied in tobacco and *A. thaliana* respectively (Zhu-Shimoni *et al.*, 1997; Rognes *et al.*, 2003). The expression of *akthr1* is subjected to spatial and temporal control in vegetative tissues, flowers and developing seeds. Using Gus reporter gene, *akthr1* gene expression was shown to be stimulated in meristematic cells and developing tissues such as, lateral buds, young leaves and developing seeds. *Akthr1* expression decreased upon maturation of leaves, stems, floral tissues, and embryo expression, and was relatively low in roots. During seed development, the expression of *akthr1* in the embryo was shown to coordinate with the initiation and onset of storage protein synthesis (Zhu-Shimoni *et al.*, 1997). *Akthr2* was also shown to expressed in meristematic cells, leaves and stamens , while the time-restricted or absent expression of the *akthr2* gene in the stem, the gynoecium and during seed formation is different from that of *ahthr1*.(Zhu-Shimoni *et al.*, 1997; Rognes *et al.*, 2003)

1.3.2.2 Homoserine dehydrogenase (HSD, EC 1.1.1.3)

HSD is the first enzyme committed to the synthesis of threonine, methionine and isoleucine. L-4-aspartyl phosphate produced by AK is then converted to L-aspartate 4-semialdehyde by aspartate semialdehyde dehydrogenase (ASADH, EC

1.2.1.11). HSD then catalyzes the conversion of L-aspartate 4-semialdehyde into L-homoserine. It competes for the same substrate with dihydrodipicolinate synthase (for lysine synthesis). Besides exists as a bifunctional threonine-sensitive AK/HSD, there is also a cytoplasmic Thr-resistant HSD isoenzymes which physiological function is still unknown (Bryan, 1980; Lea *et al.*, 1985).

1.3.2.3 Dihydrodipicolinate synthase (DHPS, EC 4.2.1.52)

Dihydrodipicolinate synthase is the first enzyme committed to the synthesis of Lys. It catalyzes the condensation of L-aspartate 4-semialdehyde and pyruvate to produce dihydrodipicolinate. In plants, DHPS is the major regulatory enzymes for Lys synthesis, and it is the most sensitive to feedback inhibition by Lys among the aspartate family amino acid biosynthetic enzymes. DHPS is about 10-fold more sensitive to Lys inhibition than the Lys-sensitive AK (Galili, 1995). On the other hand, the *E. coli* DHPS is about 100-fold less sensitive to Lys inhibition than plant DHPS (Galili, 1995).

Two genes encoding DHPS, *dhps1* (Vauterin *et al.*, 1999) and *dhps2* (Craciun *et al.*, 2000; Sarrobert *et al.*, 2000), were isolated from *A. thaliana*. While DHPS enzyme is feedback inhibited by lysine, the expression of *dhps1* was unaffected by external Lys application (Vauterin *et al.*, 1999). It was suggested that expression of *dhps1* exerts effect under normal growth and development, while its enzymatic

regulation may act to prevent accumulation of potentially toxic Lys in the organelles (Vauterin *et al.*, 1999). The expression of *dhps2* was detected in meristem, vasculature, root tips, anther, developing pollen, carpels and developing seeds (Craciun *et al.*, 2000)

1.3.2.4 Lysine α-ketoglutarate reductase (LKR, EC 1.5.1.7)

LKR is the first enzyme involved in lysine catabolism. It catalyzes the formation of saccharopine from Lys and α-ketoglutarate. The saccharopine produced is then converted to α-amino adipic semialdehyde and Glu by saccharopine dehydrogenase (SDH, EC 1.5.1.9). LKR and SDH enzyme exist in both bifunctional LKR/SDH polypeptide and separate monofunctional isoenzymes (Azevedo *et al.*, 2006).

The LKR/SDH enzyme is encoded by a bifunctional *LKR/SDH* gene, which also encodes the monofunctional LKR and monofunctional SDH isoenzymes. The expression of LKR/SDH is regulated transcriptionally and post-translationally. The LKR/SDH gene is highly expressed in floral organs and developing seeds, while the expression patterns in developing seeds differ between moncots and dicots (Galili, 2002). In *A. thaliana*, *LKR/SDH* gene is expressed in ovarian tissues, developing embryos and the outer layers of endosperms (Tang *et al.*, 1997a), while maize LKR/SDH gene is expressed predominantly in outer endosperm layers but barely

detected in embryos (Kemper *et al.*, 1999). The expression of LKR/SDH is not only subjected to developmental control but also subjected to stimulation by osmotic and salt stresses (Galili, 2002).

Enzymatic studies shown that the monofunctional LKR has nearly 10-fold lower K_m for lysine than bifunctional LKR/SDH, and the V_{max} is significant higher than the bifunctional counterpart (Galili, 2002; Tang *et al.*, 2002). *In vitro* study shown that the LKR activity of the bifunctional enzyme is regulated by the ionic strength of incubation assay, while the monofunctional LKR is unaffected (Galili, 2002). These results suggested that monofunctional LKR is a highly efficient enzyme, while the bifunctional LKR/SDH is a highly regulated enzyme (Galili, 2002).

Previous studies shown that increase in Lys production will increase activity LKR/SDH, resulting in increased levels of Lys catabolic products (Karchi *et al.*, 1994; Falco *et al.*, 1995; Mazur *et al.*, 1999). These results demonstrated the importance of Lys catabolism in regulating the Lys level.

1.3.2.5 Threonine synthase (TS, EC 4.2.3.1)

TS is the last enzyme for Thr synthesis and catalyzes the conversion of o-phospho-L-homoserine to L-threonine. The homoserine produced by HSD is converted

to o-phospho-L-homoserine by homoserine kinase (HK). The O-phospho-L-homoserine is a common substrate for TS and cystathionine γ-synthase (CGS) and will lead to the formation of Thr and cystathionine (will form Met in subsequent reactions), respectively. Thus, the competition between TS and CGS for O-phospho-L-homoserine will determine the metabolic flux of Asp into Thr and Met. Previous *in vitro* study suggested that the affinity of O-phospho-L-homoserine to TS is much higher than that to CGS (Curien *et al.*, 1998; Ravanel *et al.*, 1998).

1.3.2.6 Cystathionine γ -synthase (CGS, EC 2.5.1.48)

CGS is the first enzyme committed to Met biosynthesis. CGS catalyzes the formation of cystathionine from O-phospho-L-homoserine and cysteine. As mentioned above, O-phospho-L-homoserine is a common substrate for TS and CGS, the competition between TS and CGS regulates Thr and Met synthesis. As the cysteine is required, Met biosynthesis is a complicated process involving both nitrogen and sulfur metabolisms. The regulation of sulfur metabolism leading to the formation of cysteine has been reviewed previously (Leustek and Saito, 1999; Saito, 2000; Grossman and Takahashi, 2001).

A. thaliana CGS mutant *mto1* accumulates over 40-fold Met in leaves, compared to wild type plants (Inaba *et al.*, 1994). The over-production of Met in the

mutant is attributed to the increased mRNA stability, due to a point mutation in first exon of *CGS* (Chiba *et al.*, 1999), suggesting that the stability of *CGS* mRNA plays a significant role in regulating Met biosynthesis.

While transgenic tobacco over-expressing normal CGS is morphologically indistinguishable from wild-type, transgenic tobacco over-expressing truncated CGS exhibits a severe abnormal phenotype and accumulates a high amount of Met catabolic products such as ethylene, carbon disulfide and dimethyl sulfide. It is suggested that the truncated region is important to regulate the level of Met and its catabolic rate. As *CGS* mRNA and protein level remained the same between transgenic plant expressing normal CGS and transgenic plants expressing truncated CGS, it is proposed that this region may confer post-translational regulation of CGS (Hacham *et al.*, 2002).

In summary, the regulation of *CGS* mRNA stability, the post-translational control of CGS protein and the activity of TS enzyme act together to control Met biosynthesis in higher plants.

1.3.2.7 Threonine deaminase (TD, EC 4.3.1.19)

TD is the first and only unique enzyme to Ile biosynthesis. It catalyzes the deamination of L-threonine to 2-ketobutyrate. There are two form of TD, one is

sensitive to Ile inhibition and is predominant in young tissues, and another is insensitive to Ile inhibition and is found in old senescing tissues (Singh, 1999).

1.4 Previous attempts to manipulate seed protein quantity and quality

Researchers have made many attempts to improve seed protein quantity and quality. Aspartate family amino acids, especially Lys and Met which are deficient in plant protein, are the major targets for seed protein improvement. Previous attempts to manipulate the seed protein contents were mainly by i) enhancing the amino acids transported from source tissues to sink tissues (seeds); ii) Redirecting the metabolic pathway to increase the synthesis or reduce the catabolism in order to increase target amino acids in sink tissues; iii) Entrapping the free target amino acids into appropriate seed storage protein.

1.4.1 Enhancement of amino acids transported from source to sink

Asn is an ideal nitrogen transporting compound from source tissue to sink tissue. The free Asn levels in high-protein maize lines and high-protein rye inbred lines are both higher than their low-protein counterparts, suggesting a correlation between high free Asn levels and high seed protein contents (Dembinski and Bany, 1991; Dembinski *et al.*, 1995). In soybean, the expression of *AS1* in leaves (especially in trifoliate leaves of young seedlings) showed a positive correlation with seed protein contents in the

soybean cultivars tested (Wan *et al.*, 2006). Study in tobacco showed that the free Asn change drastically during seed development (Karchi *et al.*, 1994), suggesting Asn plays an important role in seed development. It is suggested that increased free Asn in siliques may act as a source of free Asp (via the enzymatic activities of ANS) for seed coats and cotyledons during seed development (Bewley *et al.*, 2000).Thus, it is an attractive idea to increase the production of Asn in source tissues so that assimilated nitrogen can be more effectively transported to sink tissues for seed protein production.

Previous study to constitutively overexpress *ASN1* gene in transgenic *A. thaliana* (predominantly in source tissues) leads to an elevation of protein contents in seeds. The mol% of total Asx (Asn + Asp) in seeds is also elevated. The increased nitrogen resources may come from the elevated transporting Asn in phloem (Lam *et al.*, 2003).

Another attempt is to introduce the *E. coli* ammonium-dependent asparagine synthetase gene (*asnA*) to transgenic plants. A patent has been filed based on this idea (Dudits *et al.*, 1991). However, study on transgenic rapeseeds with the *E. coli asnA* shown no effects on seed yield, total dry matter, or nitrogen contents in both straw and seeds (Seiffert *et al.*, 1999).

1.4.2 Redirection of metabolic pathways to increase target amino acids

The aspartate family amino acid pathway is tightly regulated by positive and negative regulations of branched-point enzymes (Azevedo *et al.*, 1997; Azevedo and Lea, 2001) and amino acid catabolism (Zhu and Galili, 2004). Redirection of the metabolic pathways to increase the synthesis and reduce the catabolism of the target amino acids were attempted to enhance the content of aspartate family amino acids. The attempts to manipulate Lys and Met contents by redirecting the metabolic pathway were summarized in Table 1.2

Table 1.2. Previous attempts to manipulate Lys and Met contents by redirecting the metabolic pathway.

Gene Construct	Source	Target	Promoter	Major result	Reference
TS mutant (*mto2*)	N.A.	*A. thaliana*	N.A.	22 folds increase in Met in young rosette leaves	Bartlem *et al.*, 2000
Antisense *TS*		Potato	Constitutive	up to 239 folds increase in leaf Met increase in Met in tubers	Zeh *et al.*, 2001
CGS	*A. thaliana*	*A. thaliana*	Constitutive	increase in Met and SMM	Kim *et al.*, 2002
	Maize	Maize	Seed-specific	5 folds increase in Met	Locke *et al.*, 1997
CGS mutant (*mto1*)	N.A.	*A. thaliana*	N.A.	increase in CGS mRNA stability 40 folds increase in leaf Met	Inaba *et al.*, 1994
Feedback insensitive AK (*lysC*)	*E. coli*	*A. thaliana*	Constitutive	increase in Thr no change in Lys	Ben-Tzvi Tzchori *et al.*, 1996
	E. coli	Tobacco	Constitutive	increase in Thr No change in Lys	Shaul and Galili, 1992
	E. coli	Tobacco	Seed-specific	13-16 folds increase in free Thr about 3 folds increase in free Met in seeds no change in free Lys increase in LKR activity	Karchi *et al.*, 1993
	E. coli	Alfalfa	Constitutive	increased in free and protein-bound Thr	Galili et al, 2000
Mutant RLT40 (partial insensitivity of lysine sensitive AK)		*A. thaliana*		6 fold increase in soluble threonine decreased Met content	Heremans and Jacobs, 1995

Feedback insensitive DHPS	*E. coli*	*A. thaliana*	Constitutive	10-100 folds increase in free Lys in whole plant decrease in Thr abnormal phenotype at early developmental stage	Ben-Tzvi Tzchori *et al.*, 1996
	E. coli	tobacco	Constitutive	increase in free Lys in leaves abnormal phenotype	Shaul and Galili, 1992
	E. coli	Maize	Seed-specific	increase in seed Lys increase in Lys catabolic products	Shaul and Galili, 1992
	Corynebacterium	Canola	Seed-specific	increase in free lysine poor germination	Falco *et al.*, 1995
Partial feedback insenstive of DHPS	Maize	Rice	Constitutive	increase free Lys in immature and mature seeds increase Lys catabolic products poor gemination rate	Lee *et al.*, 2001
	Maize	rice	Seed-specific	increased Lys level in immature seeds but not mature seeds increase Lys synthesis and catabolism	Lee *et al.*, 2001
Feedback insensitive AK and DHPS	*E.coli lysC* and *dapA*	tobacco	Constitutive	increase in free Lys increase in free Thr	Shaul and Galili, 1993
	E. coli dapA and *Corynebacterium lysC*	soybean	Seed-specific	200 folds increase in free Lys in seeds 5 folds increase in total Lys poor germination	Falco *et al.*, 1995

opaque-2 mutant	N.A	Maize	N.A	Decrease in LKR-SDH activity Increase in seed Lys Decrease in yield and increase susceptibility to pathogen	Azevedo *et al.*, 1990
opaque-2 / *ask1* double mutant	N.A	Maize	N.A	Increase in free and protein-bound Lys decrease zein compared to opaque-2 mutant	Azevedo *et al.*, 1990 Brennecke *et al.*, 1996
LKR/SDH T-DNA insertion mutant	N.A	*A. thaliana*	N.A	6% increase in lys in seed albumins	Zhu *et al.*, 2001
	N.A	Maize	N.A	increase in Lys and Lys catabolic products Increase in Lys catabolic products	Falco, 2001
Feedback insensitive DHPS *and* LKR/SDH mutant	*E. coli dapA*	*A. thaliana*	N.A	80 fold increase in free Lys in seeds 4.3 fold increase in total Lys content poor germination	Zhu and Galili, 2003
Feedback insensitive DHPS *and* RNAi LKR/SDH	*E. coli dapA* and *A. thaliana LKR/SDH*	*A. thaliana*	Seed-specific	Increase in free Lys in seeds Increase in total Lys Increase in free Met in seeds Increase in total Met	Zhu and Galili, 2004

1.4.2.1 Production of aspartate by Aspartate Aminotransferase (AAT)

Asp is the common precursor for aspartate family amino acids. To enhance the aspartate family amino acids in seeds, a strategy is to increase the level of Asp. Aspartate aminotransferase (AAT) catalyzes the formation of Asp and α-ketoglutarate from oxaloacetate and Glu. The manipulations of AAT by transgenic approaches at beginning stage and there are only a few attempts to increase AAT activities. Transgenic tobacco lines expressing mitochondrial and cytosolic AAT of *Panicum miliaceum* have been generated and shown to increase AAT and phosphoenolpyruvate carboxylase activities (Sentoku *et al.*, 2000). However, the effects of such manipulations on nitrogen metabolism and aspartate family amino acids have not been addressed. A later research have generated transgenic *A. thaliana* constitutively express soybean *AAT5* gene, which encodes a chloroplastic AAT, and the free amino acid level in seeds were analyzed. The transgenic lines have increased level of free glycine (Gly), alanine (Ala), Asn and Gln in seeds, but the level of Asp and aspartate-derived essential amino acids do not increase. It was proposed that the Asp overproduced by AAT is converted to Asn in consumption of toxic ammonium ions, and the increased Gln maybe due to the reverse reaction of AAT (Murooka *et al.*, 2002).

1.4.2.2 Deregulation of AK to increase the common substrate for all essential aspartate family amino acids

AK is the first enzyme in the aspartate family pathway and is committed to the synthesis of aspartate family amino acids. Monofunctional AK is subjected to feedback inhibition of Lys and SAM, while bifunctional AK/HSD is subjected to Thr inhibition. As AK is committed to the synthesis of essential aspartate family amino acids, many studies attempted to deregulate the control of AK by feedback inhibition. Several attempts were made to express, in a constitutive or seed-specific manner, a mutant allele of the *lysC* gene (encoding AK that is insensitive to lysine and threonine) from *E. coli* to transgenic *A. thaliana* (Ben-Tzvi Tzchori *et al.*, 1996), tobacco (Shaul and Galili, 1992; Karchi *et al.*, 1993), and alfalfa (Galili *et al.*, 2000). These transgenic plants exhibited increased level of free Thr but no increase in free Lys. One attempt has resulted in a slight increase in free Met (Karchi *et al.*, 1993). In *A. thaliana* mutant RLT 40, which expresses a partial lysine insensitive enzyme, Thr accumulation is accompanied by a decrease of Met (Heremans and Jacobs, 1995).

1.4.2.3 Inhibition of TS and enhancement of CGS to increase Met biosynthesis

Met is one of the major targets for seed protein manipulation. Met biosynthesis involves both nitrogen and sulfur metabolisms. The manipulations of sulfur metabolism will not be discussed here. Competition of TS and CGS determines the

metabolic flux into Thr and Met. Inhibition of TS and reduction of CGS are two strategies to improve Met content.

1.4.2.3.1 Inhibition of TS

As the affinity of O-phospho-L-homoserine to TS is much higher than that to CGS (Curien *et al.*, 1998; Ravanel *et al.*, 1998),inhibition of TS to redirect the metabolic flux from Thr to Met can enhance the synthesis of Met. In an *A. thaliana* TS mutant, *mto2*, the activity of threonine synthase is greatly impaired by a point mutation. There is a 22 fold increase of Met in young rosette leaves (Bartlem *et al.*, 2000). Moreover, transgenic potato expressing antisense TS constitutively shows an increase of Met contents up to 239 folds in leaves, when compared to the control (Zeh *et al.*, 2001). However, the Thr level remains unchanged in these transgenic plants.

1.4.2.3.2 Enhancement of CGS

Another approach to increase Met production is by enhancing the activity of CGS. In the *A. thaliana* CGS mutant, *mto1*, the Met-dependent degradation of CGS mRNA is prohibited, and there is an accumulation of 20 fold more free Met in the mutant plant when compared wild type plants (Inaba *et al.*, 1994; Chiba *et al.*, 1999). Moreover, transgenic plants overexpressing CGS exhibit enhanced levels of free and total seed Met contents. However, the enhancement of Met seems to vary among

different plant species, different tissues, and different developmental stages (Tarczynski *et al.*, 2001; Hacham *et al.*, 2002; Kim *et al.*, 2002).

Manipulations of metabolic steps downstream from CGS do not seem to be useful for manipulation of Met content. Overexpression of cystathionine β-lyase (CBL) in potato (Maimann *et al.*, 2001) and *A. thaliana* (Gakiere *et al.*, 2000) does not increase Met levels. On the other hand, although underexpression of SAMS in tobacco leads to several hundred folds increase of free Met, such disruption in SAM biosynthesis causes major defects in plant morphology (Boerjan *et al.*, 1994) This is probably due to the important roles of SAM as a primary methyl-group donor and also the precursor for metabolites such as ethylene, polyamines and vitamin B1 (Hesse and Hoefgen, 2003)..

1.4.2.4 Deregulation of DHPS and reduction of lysine catabolism to increase lysine content

Enrichment of Lys is an important target for crop improvement since its contents is generally low in cereals which are major staple food for human and feed to domestic animals. As discussed above, deregulation of the feedback control of aspartate kinase alone cannot increase the level of Lys. The accumulation of Lys is mainly limited by: (i) Lys-mediated feedback inhibition of dihydrodipicolinate

synthase (Negrutiu *et al.*, 1984; Galili, 1995); (ii) Lys catabolism by LKR (Falco *et al.*, 1995; Karchi *et al.*, 1995; Mazur *et al.*, 1999); and (iii) defects in growth and development caused by excessive free Lys (Shaul and Galili, 1992; Falco *et al.*, 1995; Mazur *et al.*, 1999; Zhu and Galili, 2003).

1.4.2.4.1 Deregulation of DHPS

To overcome feedback inhibition of dihydrodipicolinate synthase, an *E. coli* dihydrodipicoline synthase gene (*dapA*) which encode DHPS that is about 100-fold less sensitive to Lys inhibition than plant DHPS (Galili, 1995), was constitutively expressed in tobacco and *A. thaliana*. Although the transgenic plants showed increased level of Lys, they also showed defects in the development of both vegetative and reproductive tissues (Shaul and Galili, 1992, 1993; Ben-Tzvi Tzchori *et al.*, 1996). In transgenic potato, while the accumulation of Lys was considerably lower, abnormal phenotypes were not observed (Perl *et al.*, 1992). On the other hand, a slight increase of free Lys in mature seeds has been reported in transgenic barley expressing *E. coli* *dapA*, accompanied with a 50% reduction of free proline (Pro) (Brinch-Pedersen *et al.*, 1996).

Seed-specific expression was employed to reduce the detrimental effects of Lys accumulation in transgenic plants expressing bacterial *dapA* gene. In transgenic

soybean and canola expressing bacterial *dapA* gene in seed-specific manner, the levels of seed lysine increased, but poor germination was observed (Falco *et al.*, 1995). In maize expressing of a bacterial *dapA* in developing embryo, increases in level of seed lysine and Lys catabolic products were observed (Mazur *et al.*, 1999).

In transgenic tobacco and soybean co-expressing bacterial *lysC* and *dapA* free lysine levels increased, this was probably due to the competition of deregulated DHPS for the common intermediate sharing by the Thr branch of the aspartate amino acid family pathway (Shaul and Galili, 1993; Falco *et al.*, 1995).

1.4.2.4.2 Reduction of Lys catabolism

Another approach to increase the Lys content is to reduce the Lys catabolism by reduction of LKR activity. Reduction the activity of LKR/SDH activity by insertional mutagenesis (Zhu *et al.*, 2001) or mutating the transcriptional activator Opaque-2 (Azevedo *et al.*, 1990; Brennecke *et al.*, 1996) decreased the Lys catabolism, and elevated levels of free and protein bound Lys were observed.

1.4.2.3.3 Deregulation of DHPS and reduction of LKR

Elevation in Lys synthesis will increase activity of the Lys catabolic enzyme LKR, and will lead to increased levels of Lys catabolic products (Karchi *et al.*, 1994; Falco *et*

al., 1995; Mazur *et al.*, 1999; Lee *et al.*, 2001). Some previous researches attempted to combine the deregulation of DHPS and reduction of LKR to increase the Lys level (Zhu and Galili, 2003, 2004). When an *A. thaliana* lysine-ketoglutarate reductase/saccharopine dehydrogenase knock-out mutant was crossed with a transgenic plant expressing a bacterial *dapA* in a seed specific manner, a 80-fold increase in free Lys and a 4.3-fold increase in total Lys (after acid hydrolysis) were observed in seeds, accompanied by an increase in Met content and changes in other amino acid pools (Zhu and Galili, 2003). However, poor germination was observed in this construct, and this was probably due to the inhibitory effects of the accumulation of free Lys or the defective postgermination Lys catabolism.

In a recent study, transgenic *A. thaliana* co-expressed a bacterial *dapA* gene and an RNAi construct of *AtLKR/SDH*, in a seed-specific manner, was generated. The deregulation of Lys biosynthesis and reduction of Lys catabolism were restricted to seed development. The transgenic plants showed a great increase in free Lys in mature seeds when compared to wild-type, *LKR* RNAi lines and seed-specific *dapA* transgenic plants. The relative level of total Lys in mature seeds increased, and the inhibitory effect on seed germination was greatly reduced (Zhu and Galili, 2004).

1.4.3 Expression of seed storage proteins to entrap the free amino acids

Another strategy for seed protein manipulation is to provide an effective sink for the target free amino acids by expressing appropriate seed storage proteins. Proteins contributed the largest pool of amino acid storage in mature seeds, thus, expression of seed storage proteins with high content of target amino acid to entrap free amino acid is an attractive strategy to improve seed protein quality. For instance, storing the increased amount of free Lys in Lys-rich seed proteins should enhance the final contents of Lys in seeds. Entrapping the free amino acids into seed storage protein is also a strategy to reduce the adverse effects caused by abnormally high concentrations of some amino acids, e.g. Lys, which are potentially toxic to plants (Perl *et al.*, 1992; Shaul and Galili, 1992; Falco *et al.*, 1995; Lee *et al.*, 2001; Zhu and Galili, 2003). Previous attempts to enhance Lys and Met contents by expression of Met-rich or Lys-rich storage proteins were summarirzed in Table 1.3.

Table 1.3. Previous attempts to enhance Lys and Met contents by expression of Met-rich or Lys-rich storage proteins.

Protein	Target plant	Promoter	Major Results	References
Brazil nut 2S albumin (BN2S)	Tobacco	French bean phaseolin	30% increase in Met level in seed proteins	Altenbach *et al.*, 1989
	Canola	French bean phaseolin	Up to 30% increase in Met in seeds	Altenbach *et al.*, 1992
	Narbon bean	French bean phaseolin	Increase in Met in seeds	Saalbach *et al.*, 1995
	Potato	CaMV35S	BN2S represent 0.2% of total tuber proteins	Tu *et al.*,1998
Brazil nut 2S albumin (BN2S) and feedback insensitive AK	Narbon bean	French bean phaseolin for AK *Vicia faba* legumin B4 for BN2S	2-2.4 folds increase in protein-bound Met	Demidov *et al.*,2003
Sunflower seed albumin (SSA)	Subterranean clover	CaMV35S	SSA accumulated up to 1.3% of total leaf extractable proteins	Khan *et al.*, 1996
	Lupin	Pea vicilin	less sulphate and more total amino acid sulphur 94% increase in total seed Met 12% reduction in seed cysteine	Molvig *et al.*,1997
	Trifolium repens	*A. thalinana* small subunit of Ru-bisco; *M. sativa* small subunit of Rubisco; CaMV35S	SSA accumulated up to 0.1% of total extractable leaf protein	Christiansen *et al.*,2000
Amaranth seed albumin (AmA1)	Potato	Granule-bound starch synthase (GBSS) CaMV35S	30-40% increase in total tuber protein content increased tuber number and tuber yield 4-8 fold increase in Lys, Met, Cys and Tyr (for GBSS) 2.5-4 fold increase in Lys, Met, Cys and Tyr (for CaMV35S)	Chakraborty *et al.*, 2000
Winged bean lysine-rich protein	Arabidopsis	French bean phaseolin	recombinant proteins accumulated up to 10% of total extractable protein	Cheng, 1999
Posphocarpus tetragonolobus Lys-rich protein	Rice	Maize Ubiquitin	up to 16.04% enhancement in seed Lys content	Gao *et al.*, 2001
De novo protein with 31% Lys and 20% Met	Tobacco	Bean phaseolin soybean beta-conglycinin	Increase in Lys level Increase in Lys level enhanced phenotypes more stable than using bean phaseolin promoter	Keeler *et al.*, 1997

Heterologous expression of the Brazil nut 2S albumin (BN2S) by French bean phaseolin promoter increased the Met contents in the seeds of tobacco, canola and soybean (Altenbach *et al.*, 1989; Altenbach *et al.*, 1992; Saalbach *et al.*, 1995; Hesse *et al.*, 2001). Seed-specific expression of Sunflower seed 2S albumin (SSA), another Met-rich storage protein, doubled the total seed Met content (Molvig *et al.*, 1997). However, due to the allergenic natures of BN2S and SSA, commercialization of transgenic crops expressing BN2S and SSA was hampered. Another Met-rich protein, Amaranth seed albumin (AMA1) which is non-allergenic in nature, and was expressed in potato, and the transgenic potato exhibited enhancement of Lys, Met, cysteine and tyrosine (Tyr) for up to 4-8 folds (Chakraborty *et al.*, 2000).

Heterologous expression of Lys-rich protein (LRP) from Wing bean was employed to improve Lys content in plants but the results are different between dicots and monocots. In *A. thaliana* expressing LRP, the recombinant protein accumulated up to 10% of total extractable seed protein, but the seed Lys content remained steady (Cheng, 1999). However, in transgenic rice expressing of LRP, there was increase in seed Lys level for up to 20% (Gao *et al.*, 2001; Liu, 2002).

In addition to naturally-occurred storage proteins, *de novo* protein sequences were also employed for seed protein improvement. Transgenic tobacco expressing of a

de novo protein with 31% lysine and 20% showed an increase in seed lysine content (Keeler *et al.*, 1997). However, the allergenicity of proteins is a consideration for using this kind of artificial proteins for seed protein improvement.

1.5 Expression of multiple transgenes in plants

1.5.1 Significance of multiple genes manipulation in seed quality improvement

Researchers have put much effort to improve the seed protein quantity and quality. Compare with transgenic plant expressing a single transgene, transgenic plants expressing a combination of two strategies showed more profound effects in Lys and Met enhancement (Demidov *et al.*, 2003; Zhu and Galili, 2004). To further explore the manipulation of Lys and Met content in seeds, generation of transgenic plants with different combination of transgenes altering the nitrogen sink-source relationship will give us new insights. To achieve this goal, systems for transformation of multiple transgenes into plants simultaneously are required.

1.5.2 Difficulties in introduction of multiple genes into plant genomes

Traditional binary vectors are generally difficult for cloning of multiple target gene cassettes due to the lack of restriction cloning sites in the multiple cloning sites (Lin *et al.*, 2003; Chung *et al.*, 2005). Other approaches to introduce multiple genes into plant genome also have their specific problems. Sexual crossing between plants

carrying different transgenes is time-consuming (Lin *et al.*, 2003), while sequential retransformation of transgenic plants is also time consuming and requires different selectable markers. For cotransformation with multiple plasmids, the transformation efficiency decreases when the number of plasmids increases.

1.5.3 Recent advances in introduction of multiple genes into plant genome

Recently, researchers developed novel systems for transformation of multiple genes with the use of site-specific recombination and rare-cutting restriction enzymes (Chung *et al.*, 2005). With the use of Cre/*loxP* site-specific recombination and rare-cutting homing endonuclease, a multigene assembly and transformation system have been developed (Lin *et al.*, 2003). The system consists of three *loxP* site containing vectors, an acceptor vector for plant transformation and two donor vectors for subcloning of target gene cassettes. After cointegration of the acceptor vector and target gene carrying donor vector, homing endonuclease is used to remove the backbone of the donor vector, which contain an unwanted *loxP* site and bacterial selective marker. As homing endonucleases have long and rarely-existed recognition site, it will not cut the backbone of the acceptor vector and the inserted target genes. By alternative use of the two donor vectors, multiple rounds of recombination can be carried out to deliver as many as ten genes into the acceptor vector for plant transformation (Lin *et al.*, 2003).

Another system using the GATEWAY™ technology to generate vector for transformation of multiple genes was also developed (Karimi *et al.*, 2005). GATEWAY™ technology is based on the site-specific recombination between two DNA molecules with complementary recombination sites and this provides a fast and reliable way for introduction of target genes into binary vectors (Karimi *et al.*, 2002). Karimi's group has developed a set of binary vectors with a recombination cloning Multisite GATEWAY™ cassette, these vectors allow the transfer of as many as three genes into the binary vectors for plant transformation (Karimi *et al.*, 2005).

There is also a system for simultaneous transfer of multiple genes into plant without using site-specific recombination. A set of auxiliary vectors with the multiple cloning sites flanked by homing endonuclease sites was developed. After construction of target gene cassettes into auxiliary vectors, the cassettes can be transferred to a binary vector by the use of appropriate homing endonucleases. This system allows as many as six gene cassettes to be transferred to plants simultaneously (Goderis *et al.*, 2002).

1.6 Global nitrogen regulators in plants

1.6.1 Global regulation of nitrogen metabolism

To improve the seed protein quantity and quality, another strategy is to manipulate the global nitrogen regulators that control the overall nitrogen status. Nitrogen is an essential element for the biosynthesis of vital biomolecules such as amino acids, proteins, and nucleic acids. Nitrogen metabolism is an important cellular process that is well monitored and regulated. In bacteria and fungi, a few sophisticated N regulatory systems have been extensively studied.

PII is a signal transduction protein that was first identified in *E. coli* and exists in all three domains of lifes (Hsieh *et al.*, 1998; Archondeguy *et al.*, 2001). In *E. coli*, PII regulates the N metabolism by regulating the key nitrogen assimilation enzyme glutamine synthetase (GS) at both transcriptional and post-translational level (Magasanik, 1988, 1993; Atkinson *et al.*, 1994). In N limiting condition, PII will be uridylylated at Tyr-51. PII-UMP promotes N metabolism by activating GS enzyme via stimulating the deadenylylation activity of ATase. The uridylylated PII is removed from NRII so that the kinase activity of NRII (Jiang *et al.*, 1998) can phosphorylate NRI which in turn activates the transcription of the *glnA* gene which encodes GS (Magasanik, 2000).Conversely, in N rich conditions, PII remains unmodified. Free PII proteins suppress N assimilation via stimulating the transferase activity of ATase and hence reducing GS enzymatic activity by increasing the degree of adenylylation on GS. It also binds to NRII to activiate the dephosphorylation activity of NRII toward NRI,

leading to a loss in NRI transcriptional activity (Magasanik, 2000). Previous studies in cyanobacteria suggested that PII regulates nitrogen metabolism by acting on the enzyme N-acetyl-L-glutamate kinase (NAGK) to regulate the arginine synthesis in response to nitrogen status (Maheswaran *et al.*, 2004). The regulation of PII protein is controlled by phosphorylation but not uridylylation in cyanobacteria (Lee *et al.*, 2000)

Another nitrogen regulatory system is the General amino acid control (GAAC) by General Control Non-inducible (GCN) system identified in yeast (Figure 1.3) and will be discussed in next section. This system responses to nitrogen starvation, activates multiple amino acid biosynthetic pathway, and synthesizes more amino acids to cope with the starvation (Wek *et al.*, 1989; Hinnebusch, 1994; Hinnebusch, 1997, 2005).

1.6.2 General amino acid control by GCN system

In yeast, amino acid starvation will trigger the GAAC and up-regulate at least 35 genes in 12 different amino acid biosynthetic pathways (Hinnebusch, 1994). GCN system is key for this kind of general amino acid control in yeast (Wek *et al.*, 1989; Hinnebusch, 1994; Hinnebusch, 1997). Amino acid starvation will lead to increased level of uncharged tRNA, facilitated by the binding of GCN1/GCN20 protein complex to GCN2 (Marton *et al.*, 1997; Garcia-Barrio *et al.*, 2000), kinase activity of GCN2

will be activated. Activated GCN2 will phosphorylate eIF2 α, which will in turn induce the translation of transcriptional activator GCN4 (Dever *et al.*, 1992). GCN4 will then bind to and act on GCN4-responsive element (*GCRE*; 5'-TGACTC-3') in the promoter region, and turn on the transcription of a number of amino acid biosynthetic gene to synthesize more amino acid and cope with the starvation(Arndt and Fink, 1986; Natarajan *et al.*, 2001). A recent research shown that GCN4 can up-regulate the expression of 539 target genes in yeast, including genes of twelve different amino acid biosynthetic pathways (with the exception of cysteine), vitamin biosynthesis, peroxisome biogenesis, glycogen metabolism, protein kinases, and transcription factors (Natarajan *et al.*, 2001).

1.6.3 General amino acid control in plants

In plants, although the mechanism of N regulation is far from clear, some putative nitrogen regulatory systems have been identified in plants. The plant PII homologs were identified in *Arabidopsis thaliana* and *Ricinus communis* (Hsieh *et al.*, 1998). PII was shown to interact with NAGK in *A. thaliana* (Burillo *et al.*, 2004) and *Oryza sativa* (Sugiyama *et al.*, 2004), and a recent research using PII knock-out mutant shown that PII regulates arginine synthesis in *A. thaliana* (Ferrario-Mery *et al.*, 2006).

Cross-pathway regulation of amino acid biosynthesis suggests the existence of general amino acid control in plants. In *A. thaliana*, blocking histidine biosynthesis by IRL1803 up-regulated the expression of eight metabolic genes involved in histidine, aromatic amino acid, lysine and purine biosynthesis (Guyer *et al.*, 1995). Moreover, mutants defective in BBMII (N'-[(5'-phosphoribosyl)-formimino]-5-aminoimidazole-4-carboxamide ribonucleotide) isomerase involved in histidine biosynthesis caused a two-fold up-regulation in total free amino acid content at the juvenile stage (Noutoshi *et al.*, 2005). These results demonstrate the existence of cross-pathway regulation of amino acid biosynthesis in plants. Another evidence of this regulation was that starvation in aromatic amino acids, branched-chain amino acid or methionine can all induce tryptophan pathway enzymes in *A. thaliana* (Zhao *et al.*, 1998).

The plant homologs of GCN key components, eIF2α (Chang *et al.*, 1999; Chang *et al.*, 2000)and GCN2 (Zhang *et al.*, 2003), were identified in wheat and *A. thaliana*, respectively. The cross-pathway regulation of amino acid biosynthesis and existence of homologs of GCN components suggested the existence of GCN system in plants. However, the physiological roles of these GCN components in plants are still unclear. As GCN system can regulate the biosynthesis of multiple amino acids in yeast, studying plant GCN system may give clues to seed protein manipulation.

1.6.4 GCN system in plants

The existence of homologs of GCN system components further supports there is a general amino acid control in plants. The plant eIF2α phosphorylation pathway was identified in wheat (Chang *et al.*, 1999; Chang *et al.*, 2000; Gil *et al.*, 2000). The yeast GCN2 can phosphorylate wheat eIF2α *in vitro* at Ser-51 (Chang *et al.*, 1999). Moreover, wheat eIF2α can functional complement yeast mutants defective in eIF2α and increase the translation of yeast GCN4 (Chang *et al.*, 2000).

The homolog of GCN2, another key component of GCN system, was identified in *A. thaliana* (Zhang *et al.*, 2003). AtGCN2 exhibits 30% overall amino acid sequence identity and 45% identity in protein kinase domain with yeast GCN2 (Zhang *et al.*, 2003). *AtGCN2* can functional complement yeast *gcn2* mutant to enable the growth of the mutants in the presence of branched-chain amino acid inhibitor and histidine inhibitor (Zhang *et al.*, 2003). Possible homologs of GCN1 and GCN20, which facilitate GCN2 activation in yeast, were also identified in *A. thaliana* (Chow, 2002; Kato *et al.*, 2004).

A key GCN system component that is still missing in plant is GCN4. Although plant GCN4 is missing, putative GCN4 motifs have also been found in promoters of some plant genes such as rice glutelin (Yoshihara *et al.*, 1996; Wu *et al.*, 1998;

Onodera *et al.*, 2001). On the other hand, the maize opaque2 can functionally complement yeast gcn4 mutant (Mauri *et al.*, 1993), and its translation of was regulated by upstream open reading as that of yeast GCN4 (Lohmer *et al.*, 1993). In maize, opaque2 was also found to interact with transcriptional co-activators GCN5 and ADA2, which are transcriptional co-activators for GCN4 in yeast (Barlev *et al.*, 1995; Kuo *et al.*, 2000; Swanson *et al.*, 2003), to modulate transcriptional activity (Bhat *et al.*, 2004). These evidences suggest that opaque2 maybe a functional homolog of GCN4, ie. a putative candidate of downstream target of eIF2α-phosphorylation pathway in plants.

Although many homologs of GCN system components exist in plants, there is no direct evidence that this system plays a role in general amino acid control in plant. The physiological role of GCN system in plants is still awaited for further investigation.

1.7 Hypothesis and specific objectives of this study

Improvement in quantity and quality of seed proteins is one of the major targets for crop improvement, and the essential aspartate family amino acids, especially lysine and methionine, are of great nutritional and economical importance. However, improvement in lysine and methionine contents in seeds is not an easy task and the results of previous attempts are not very promising. We hypothesized that the

accumulation of aspartate family amino acids is limited by an insufficient source, an ineffective sink and the tight regulation of aspartate family amino acid metabolism.

In this study, five transgenic lines of *A. thaliana* with altered sink-source of aspartate family amino acids are characterized to study their performance on seed protein improvement.

1) 35S-*ASN1* transgenic plants: D2d6

In this line, *ASN1*, encoding asparagine synthease, is constitutively expressed under 35S promoter. The overexpression of the asparagine synthetase increases the synthesis of Asn, which is an ideal N transporting compound, and lead to an enhanced transport of Asn from source tissues to sink tissues. The increased level of Asn in developing siliques can be converted to aspartate, by the action of asparaginase, to provide an enhanced source for synthesis of aspartate family amino acids.

2) phas-*PN2S* transgenic plants: C-6-3

PN2S is a Met-rich protein from paradise nut. Seed-specific expression of this Met-rich protein, using phaseolin promoter, provides an effective sink for free Met in seeds.

3) phas-*LRP* transgenic plants: 4-1-7

LRP is a Lys-rich protein from wing bean. Seed-specific expression of this Lys-rich protein, using phaseolin promoter, provides an effective sink-for free Lys in seeds.

4) phas-*MetL* transgenic plants: 6-6-1

In *A. thaliana*, bifunctional enzymes aspartate kinase-homoserine dehydrogenase is feedback inhibited by threnoine. *MetL* encodes *E. coli* AK-HSDII which is insensitive to feedback inhibition (Azevedo et al, 1997). In the transgenic plants, *metL* is expressed in seed-specific manner by phaseolin promoter.

5) phas-*dapA*/phas-*LRP* transgenic plants

In this line, both *dapA*, encoding dihydrodipicolinate synthase, and *LRP* are expressed under phaseolin promoter. In *A. thaliana*, dihydrodipicolinate synthase, which catalyzes the rate-determining step of lysine biosynthesis, is subjected to feedback inhibition by lysine. Seed-specific expression of feedback insensitive *E. coli dapA*, enhances Lys biosynthesis in seeds. The increased level of free Lys synthesized is incorporated into LRP.

I also attempt to generate transgenic *A. thaliana* with a combination of altered sink-source relationship of aspartate family amino acids to enhance lysine content in seeds. The strategy is 1) to provide an enhanced source by overexpression of *ASN1* to

enhance the asparagine transported to seeds and seed-specific expression of *E.coli ansB* to enhance the conversion of asparagine to aspartate; 2) to provide an effective sink of lysine by reduction of lysine catabolism by seed-specific expression of an antisense fragment of *LKR* and seed-specific expression of wing bean lysine-rich protein (LRP) to entrap free lysine; 3) to deregulate the feedback inhibition of rate-determining step of lysine synthesis by seed-specific expression of feedback insensitive *E. coli dapA*.

In bacteria and fungi, there are sophisticated N regulatory systems to monitor the N status and regulate the N metabolism. For instance, the GCN system in yeast monitors the N status of the cell and regulates the biosynthesis of amino acids. Cross-regulation of amino acids and identification of homologs of GCN system components suggest the existence of homologous system in plants. We hypothesized that the GCN system regulates the amino acid biosynthesis and control the accumulation of seed proteins in *A. thaliana*. In this study, transgenic *A. thaliana* overexpressing *AtGCN2* is studied to investigate the role of GCN2 in regulation of amino acid biosynthesis and explore the possibility of improving seed protein by manipulation of GCN2.

Chapter 2 Materials and methods

2.1 Materials

2.1.1 Vectors, bacterial strains and plants

The *Escherichia coli* strain DH5α was used as the host for gene cloning unless stated otherwise. The *Agrobacterium tumefaciens* strain GV3101 containing helper Ti-plasmid pMP90 was employed for plant transformation. *Arabidopsis thaliana* ectotype Columbia-0 is the seed stock in our laboratory. Col-0 and transgenic line overexpressing *ASN1* (D2d6) were used as plant host for *Agrobacterium*-mediated transformation. A list of vectors, bacterial strains and plant hosts employed in this research was shown in Table 2.1.

Table 2.1 Vectors, bacterial strains and plant hosts used.

Vectors	Description	Refernces
pCAMBIA1301/Gt1P-SP-LRP-NosT	Plant expression vector carrying *LRP* gene fused with rice glutelin promoter and signal peptide, and nopaline synthase terminator	From Dr. Samuel Sai-Ming Sun and Dr. QiaoQuan Liu
pTZ/phas-His30	Plasmid carrying His30 fused with French bean phaseolin promoter and terminator	From Dr. Samuel Sai-Ming Sun and Dr. Li Chen
pBK-*ansB*	Plasmid carrying *ansB* gene	Lab stock From Mr. Johann Ma
pTZ/phas-TP-*dapA*	Plasmid carrying *dapA* fused with chloroplastic transit peptide, inserted between phaseolin promoter and terminator	Lab stock From Mr. Piu Wong
pYLTAC747H	Plant expression vector for multigene assembly, carrying kanamycin-resistant gene as bacterial selective marker and hygromycin-resistant marker gene as plant selective marker	Lin *et al.*, 2003
pYLVS	Donor vector for subcloning of target for mutligene assembly, carrying chloramphenicol-resistant gene as bacterial selective marker	Lin *et al.*, 2003
pYLSV	Donor vector for subcloning of target for mutligene assembly, carrying chloramphenicol-resistant gene as bacterial selective marker	Lin *et al.*, 2003
pYLVS/GT1P-SP-LRP-NosT	Donor vector pYLVS carrying *LRP* fused with *glutelin1* promoter and signal peptide	This work
pYLSV/phasP-TP-dapA-phasT	Donor vector pYLSV carrying *dapA* fused with chloroplastic transit peptide, inserted between phaseolin promoter and terminator	This work
pYLVS/phasP-ansB-phasT	Donor vector pYLVS carrying ***ansB*** , inserted between phaseolin promoter and terminator	This work
pYLSV/phasP-TP-antiLKR-phasT	Donor vector pYLVS carrying an antisense fragment of *LKR* , inserted between phaseolin promoter and terminator	This work

pYLTAC747H/*LRP-dapA-ansB*-anti*LKR*		This work
Bacterial Strain	**Description**	**References**
Escherichia coli DH5α	A recombinant-deficient amber suppressing strain used for regular cloning	Lab stock
Agrobacterium tumefaciens GV3101	For *A. thaliana* transformation	Koncz and Schell, 1986
Escherichia coli NS3529	A bacterial strain expressing Cre recombinase for recombination during multigene vector manipulation	Sauer and Henderson, 1998; Lin, *et al.*, 2003
Plant hosts	**Description**	**References**
Columbia-0	*A. thaliana* wild-type ecotype	Lab stock
D2d6	Homozygous transgenic line constitutively overexpressing *ASN1*	Lab stock
C-6-3	Homozygous transgenic line expressing Paradise nut 2S albumin (PN2S) in a seed specific manner	From Dr. Samuel Sai-Ming Sun and Dr. Li Chen
4-1-7	Homozygous transgenic line expressing Wing bean *LRP* in a seed specific manner	From Dr. Samuel Sai-Ming Sun and Mr. Man Kin Cheng
6-6-1	Homozygous transgenic line expressing *E. coli metL* in a seed specific manner	From Dr. Samuel Sai-Ming Sun and Dr. Li Chen
8-1-1	Homozygous transgenic line expressing *E. coli dapA* and *LRP* in a seed specific manner	Lab stock
GCN2A	Homozygous transgenic line constitutively overexpressing *AtGCN2*	Lab stock

2.1.2 Chemicals and reagents used

The major chemicals and reagents used in this research are listed in Appendix I.

2.1.3 Buffer, solution, gel and medium

Unless otherwise stated, buffer, solution and medium were prepared according to the formulation listed in Appendix II.

2.1.4 Commercial kits used

The major commercial kits used in this research are listed in Appendix III.

2.1.5 Equipments and facilities used

All equipments and facilities were provided by Department of Biology, CUHK. An inventory is shown in Appendix IV.

2.2 Methods

2.2.1 Molecular techniques

2.2.1.1 DNA gel electrophoresis

Agarose gel was prepared by heat-dissolving 10 mg/ ml agarose powder in Milli-Q H_2O using a microwave. After cooling down to hand-hot, 50X TAE buffer (20ul/ml of Milli-Q H_2O) and 1μl 1mg/ ml ethidium bromide was added before pouring onto a gel caster. DNA samples in 1X bromophenol blue loading dye were normally loaded onto 1% agarose gels. The gel electrophoresis was run in 1X TAE buffer at 80 V for 20 minutes to 2 hours.

2.2.1.2 PCR technique

The PCR reaction mixture contained 0.2 μM of each primer, 1X PCR buffer with 1.5mM $MgCl_2$, 0.2mM dNTPs and 0.5 Unit Taq DNA polymerase were reacted in a final volume of 50μl. The PCR cycle profile was as follows: 94°C for 5 minutes before 25 cycles of 94°C for 30 seconds, appropriate annealing temperature for 30 seconds, 72°C for 1 minutes to 2 minutes (depending on the length of target fragment to be amplified) and an 10-minute additional extension step at 72°C when all cycles were completed.

2.2.1.3 Restriction digestion

DNA sample, appropriate restriction enzyme (2 units per μ g of DNA sample), 1g/L Bovine serum albumin (BSA) and 1X restriction buffer (as recommended in the company product notes) were mixed well in a 1.5 ml microcentrifuge tube. The digestion reaction mixture was incubated at 37°C for 3 hours to overnight.

2.2.1.4 Ligation (for sticky-end ligation)

DNA fragments and vectors with compatible ends (in molar ratio of insert to vector of 3 to 1), 1g/L Bovine serum albumin (BSA), 1mM ATP and 3 units T4 DNA ligase (NEB) in 1X T4 DNA ligase buffer (NEB) were mixed well in a 1.5 ml microcentrifuge tube and the reaction mixture was incubated at 16°C overnight.

2.2.1.5 DNA purification

DNA purification was performed using the Bio-Rad Prep-A-Gene DNA Purification kit. The procedures were according to the commercial manuals except sterilized deionized water instead of elution buffer was used to elute the DNA.

2.2.1.6 DNA sequencing

The ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer 402078) was used. After cycle sequencing PCR, the single-strand

DNA products were purified. 25µl absolute ethanol, 1 µl 3M sodium acetate, pH5.2 and 0.5µl glycogen were added to the PCR products. The mixture was kept in -20°C for 30 minutes and then centrifuged at 14,000g for 30 minutes. The DNA pellet was washed with 70% ethanol and then dried by a SpeedVac System (Labconco 79840-01). The pellet was then resuspended in 12.5µl Hi-diformamide (Perkin Elmer), denatured at 95°C for 2 minutes and placed on ice immediately. The sample was then loaded to the Genetic Analyzer ABI prism 3100 and raw data of the sequencing reaction were collected by ABI PRISM 3100 Genetic Analyzer Data Collection software and analyzed by ABI PRISM 3100 Genetic Analyzer Sequencing Analysis software.

2.2.1.7 Transformation of competent *E.coli* cells

The plasmid DNA was transformed into DH5α competent cells via heat shock calcium chloride mediated transformation. The plasmid DNA was added to an aliquot of 0.1 ml pre-chilled competent cells. The mixture was incubated on ice for 10 minutes and subjected to a heat shock of 42°C for 2 minutes, 0.5 ml LB broth were immediately added to the cell after heat shock and the mixture was incubated at 37°C for 1to 2 hour with shaking at 250 rpm (Orbital shaker, Lab. line 4628-1) to rescue the cells. The recovered cells were spread on LB agar plate with appropriate antibiotics for selection and incubated at 37°C overnight.

2.2.1.8 Preparation of plasmid from bacterial cells

Plasmid DNA was isolated using the Wizard plus minipreps DNA purification kit (Promega). The procedures were according to the commercial manuals.

2.2.1.9 Transformation of competent *Agrobacterium tumefaciens* cells

Recombinant plasmid was transformed into the disarmed *Agrobacterium* strain GV3101 (Merritt *et al.*, 1999) by electroporation using a Gene Pulser apparatus (BioRad GenePulser, Model No. 165-2076). An aliquot (0.04 ml) of competent cells (GV3101) was thawed and placed on ice. 1ul of recombinant DNA (10-100ng) was mixed with the competent cells in a pre-chilled 0.2 cm electroporation cuvette (BioRad). The suspension was shaken to the bottom of the cuvette and was sat on ice for 5 minutes. After drying the surface of cuvette with tissue paper, the cuvette was inserted into the Gene Pulser apparatus. Electroporation was performed at 25 μ F, 2.5kV, 600 Ohms. After a pulse, 1 ml SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) was added to resuspend the cell with a sterile Pasteur pipette. The cell suspension was transferred to a 2 ml microcentrifuge tube and was shaken at 28°C for 2 hours. 100 μ l of cells was spread on YEP plate supplemented with kanamycin

(50mg/L), rifampicin (50 mg/L) and gentamycin (25mg/L). The plate was incubated at 28°C for 2 days.

2.2.1.10 DNA extraction from plant tissue (Small-scale)

The protocol of isolation for plant genomic DNA was modified from Doyle and Doyle (1987). Small plant tissue was first frozen and ground in liquid nitrogen before homogenized with 0.5ml 2X CTAB extraction buffer (0.1M Tris-HCl (pH8), 1.4M NaCl, 0.1M EDTA (pH8), 2% (w/v) CTAB, 1% (w/v) Polyvenylpyrolidone and 0.2% β- mercaptoethanol). The extract was then incubated at 60°C for 30 minutes before centrifuged at 3000g at room temperature for 10 minutes. Aqueous layer was transferred to a new tube and extracted with one volume of phenol: chloroform: isoamylalcohol (PCI) (25:24:1) once and one volume of chloroform: isoamylalcohol (CI) (24:1) for twice. One-tenth volume of 3M sodium acetate (pH 5.2) and two volumes of absolute ethanol were added to precipitate the nucleic acid. The mixture was kept at -20°C overnight. After centrifugation at 14,000g for 30 minutes and discarding the supernatant, the pellet was washed with washing buffer (76% EtOH with 0.01M NH_4Oac), then with 70% ethanol and dried by a SpeedVac System (Labconco 79840-01). Finally, the pellet was resuspended in sterilized Milli-Q H_2O

supplemented with 1µg/ ml RNaseA and incubated at 37°C for 30 minutes to remove RNA (Doyle and Doyle, 1987).

2.2.1.11 RNA extraction from plant tissue

Plant RNA extraction protocol was modified from a standard protocol (Ausubel *et al.*, 1995). Approximately 1 g plant tissue for RNA extraction was harvested and immediately frozen and ground in liquid nitrogen. 5 ml PCI was used to homogenize the sample and 5 ml extraction buffer was added. After mixing by vigorous shaking, the two phases were separated by centrifugation at 4°C, 8000 rpm for 5 minutes. The aqueous portion of the sample was re-extracted once with PCI, then with CI. One-tenth volume of 3M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol were added to the resulting aqueous layer and the sample was stored at -20°C overnight to precipitate the nucleic acids. After centrifugation at 4°C, 8000 rpm for 20 minutes (rotor F34-6-38: Centrifuge 5810R, Eppendorf), supernatant was discarded and the nucleic acid pellet was resuspended with 1 ml 3M sodium acetate, pH 5.6 and the suspension was transferred to a 1.5ml microcentrifuge tube. After centrifugation at 13000 rpm for 30 minutes, mRNA and rRNA were precipitated and tRNA and DNA remained in the supernatant. After repeating the 3M sodium acetate pH5.6 extraction one more time (using 0.5ml this time), the pellet was then resuspended in 0.4ml 0.3M

sodium acetate pH5.6 and the RNA was precipitated by adding 1ml 100% ethanol and kept at -20°C overnight. After centrifugation at 13, 000 rpm (Refrigerated centrifuge 5810R, Eppendorf 03463) for 30 minutes and removal of supernatant, the RNA pellet was air-dried before resuspended in DEPC-treated deionized water.

2.2.2 Growth conditions of *A. thaliana*

2.2.2.1 Surface sterilization of *A. thaliana* seeds

Seeds of *A. thaliana* were put into 1.5 ml microcentrifuge tube and were surface sterilized with 1 ml Chlorox (hypochlorite, 5.25%) for 3 minutes with shaking and vortexing. The seeds were then spun down and chlorox was removed. 1 ml of sterile water was added. After shaking and vortexing for 1 minute, the seeds were spun down and the water was removed. The washing steps were repeated for two more times with sterile water. 0.5 ml of sterile water was used to suspend the seeds.

2.2.2.2 Growing *A. thaliana*

Unless otherwise stated, plant materials were grown in the following conditions. Surface sterilized Seeds were sown on Murashige and Skoog (Murashige and Skoog, 1962) basal medium (pH adjusted to 5.7 with KOH; Invitrogen, Carlsbad, CA) containing 1% (w/v) Suc and 0.9% (w/v) agar. The seeds were then allowed to imbibe on Murashige and Skoog agar plates at 4°C in the dark for 2 days before being

transferred to environmentally controlled growth chambers at 22°C with 70% relative humidity under the light/ dark cycle of 16 hours light and 8 hours dark. 10-day-old seedlings were transferred to Metro-Mix 200 soil (Hummert, St. Louis) for further growth. Different lines were transferred to separate pots and subirrigated using a common tray. Water was added to the tray but not to the individual pots to ensure even distribution of water. Regular day/night cycle was set to a day length (illumination of 80 μE at 22°C) of 16 h and a dark period (at 20°C) of 8 h.

2.2.3 Characterization of transgenic *A. thaliana* with altered sink-source relationship

2.2.3.1. Determination of amino acid contents in seeds

Plants were grown as described in **Section 2.2.2.2** and allowed to shed seeds. The thoroughly air-dried seeds from each plant were collected independently. For each line, seeds of three or more plants were collected and analyzed. Analysis and preparation of acid-hydrolyzed seeds were performed by the Australian Proteome Analysis Facility (Macquarie University, Sydney, Australia). About 70 to 100 mg of air-dried seeds underwent liquid phase acid hydrolysis in 6 M HCl at 110°C. After being dried and derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, samples were analyzed by RP-HPLC (Waters 2690 Alliance, Waters, Milford, MA).

2.2.3.2. Expression study of developing siliques of transgenic lines

2.2.3.2.1 Tagging siliques of different developmental stages

To determine the age of siliques, fully open flowers were tagged every two days and the seed age were determined in terms of days after flowering (DAF). After 12 days, tagged siliques were collected for RNA extraction. A time-course that spanned from 4DAF to 12DAF was performed.

2.2.3.2.2 Extraction of silique RNA

For the silique, RNA was prepared using the same protocol except that 0.5 volume of high salt precipitation solution (0.8M Sodium citrate, 1.2M NaCl) and 0.5 volume of isopropanol were used instead of ethanol to precipitate the nucleic acids in the first precipitation step.

2.2.3.2.3 cDNA synthesis

To synthesize cDNA, total RNA from each sample was first treated with DNaseI (Invitrogen) at 24°C for 30 min. The treated RNA was checked for genomic DNA contamination by means of PCR using the actin primers. The treated RNA was reverse transcribed using 18-mer oligo-dT and SUPERSCRIPT^{TM}III RNase H, Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

2.2.3.2.4 Real-time PCR

Real-time PCR amplification of cDNA was performed using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems). Reactions were carried out in a 96-well plate with dome cap in a 20μl reaction volume containing 10μl SYBR Green PCR Master Mix (Applied Biosystems), and 0.3μM each of the forward and reverse primer. All reactions were repeated independently for at least three times to obtain consistent results. Data was normalized using actin. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The changes in mRNA expression level were tested by an independent *t*-test, with a significance level at $p \leq 0.05$ level.

Real-time PCR were carried out to study the expression of the following genes with the primers employed were listed below.

Primers used in real-time PCR:

actin forward GGTAACATTGTGCTCAGTGGTGG,

actin reverse AACGACCTTAATCTTCATGCTGC;

akthr1 forward ATAGCTGGTCATGCAACCGAAT

akthr1 reverse TCCATCCAAGTGCAGTCCAA;

akthr2 forward AACGCAGTCTCTGAGGCATTG,

akthr2 reverse CGACTGCAGCTAAGATGCTACAG;

AtGCN2 forward CAGGCGGACTTTGACATTGTT,

AtGCN2 reverse AGATCCGCGATGAAAGATGTG;

dhps2 forward TCTTCAATTCCCCTGTCCTAAGC;

dhps2 reverse CTTTGGGAGAGACCCATTTCG;

CGS forward CAACAGAATTCGACCGCTTTTA;

CGS reverse TCATGTTCGGGATGACTTGGA

2.2.4 Characterization of transgenic *A. thaliana* overexpressing *GCN2*

2.2.4.1 Gene expression study of vegetative tissues by real-time PCR

Surface sterilized seeds were sown on Murashige and Skoog agar plates. The seeds were then allowed to imbibe on Murashige and Skoog agar plates at 4°C in the dark for 2 days before being transferred to environmentally controlled growth chambers at 22°C under the light/ dark cycle of 16 hours light and 8 hours dark for 11 days. 11-day-old seedlins were transferred to Murashige and Skoog agar plates with or with 10 μM azaserine and treated for 3 days. 14-day-old seedlings were collected for RNA extraction as stated in **Section 2.2.1.11**. Then, cDNA were synthesized as stated in **Section 2.2.3.2.2**. Real-time PCR of genes listed in **Section 2.2.3.2.3** were performed.

2.2.4.2 Gene expression study of developing siliques by real-time PCR

Wild-type Col-0 and *GCN2* were grown as mentioned in **Section 2.2.2**. Siliques of 10DAF were collected for expression study with same method as stated in **Section 2.2.3.2**.

2.2.5 Making transgenic *A. thaliana*

2.2.5.1 Cloning of multigene construct

2.2.5.1.1 Subcloning of target genes into donor vectors

2.2.5.1.1.1 Cloning of LRP into donor vector VS

Cloning of LRP cassette into donor vector pYLVS was illustrated into Figure 2.2. Plant expression vector pCAMBIA1301 carrying LRP gene fused with rice glutelin promoter and signal peptide, and nopaline synthase terminator was employed. The Gt1P-SP-LRP-NosT fragment was released from pCAMBIA1301 vector by HindIII and *EcoRI* digestion. The released LRP cassette was ligated to *HindIII* and *EcoRI* digested donor vector pYLVS

ATG GGT GTT TTC ACA TAT GAG GAT GAA ACC ACT TCA CCA GTG GCT

CCT GCT ATC CTT TAC AAA GCA ATA GTT AAA GAT GCT GAT AAC ATC

TTT CCA AAG GCT GTT GAT TCC TTT AAG AGT GTT GAA ATT GTT GAG

GGA AAT GGT GGT CCT GGA ACC ATC AAG AAG ATC TCT TTT GTT GAG

GAT GGG GAA AGC AAG TTT GTG TTG CAC AAG ATT GAG TCA ATT GAT

GAG GCT AAT TTG GGA TAC AGC TAC AGC ATA GTT GGT GGT GCT GCT

TTG CCA GAC ACA GTG GAG AAG ATT ACA TTT GAG TCC AAA TTG AGT

GCT GGA CCT TCT GGA GGC TCT GTT GGG AAA CTC ACT GTG AAA TAC

CAA ACC AAA GGA GAT GCT GAG CCC AAT GAA GAG GAA CTC AAA GTT

GGC AAA GCC AAG GGT GAT GCT CTC TTC AAG GCT GTT GAG GCT TAC

CTT TTG GCC CAT CCT GAA TAC AAT TGA

Figure 2.1. The nucleotide sequence of *LRP* gene.
The underlined ATG and TGA represent the start and stop codon respectively.

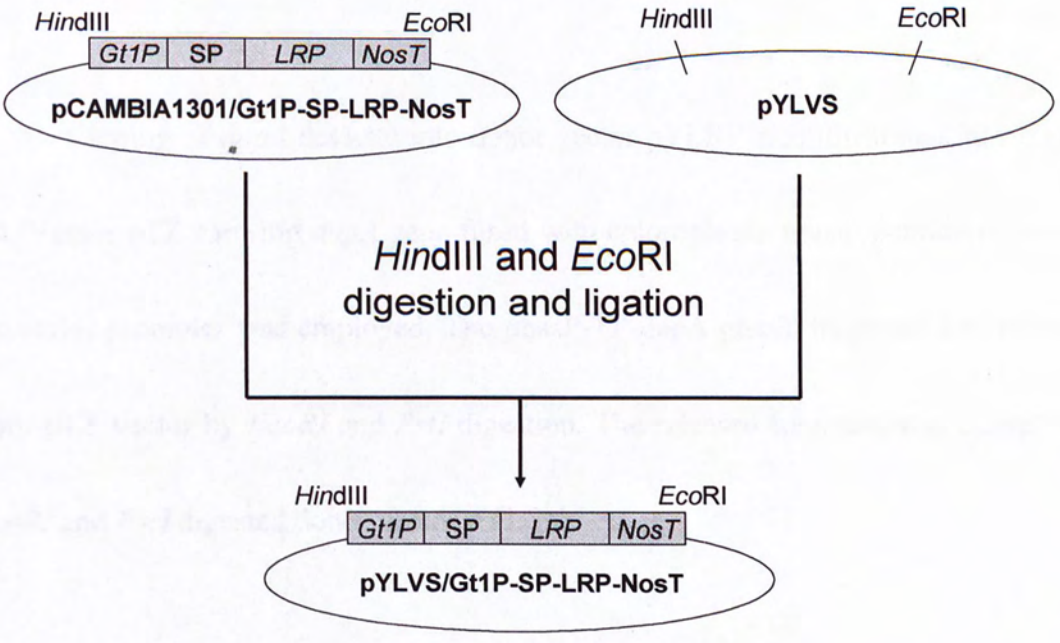


Figure 2.2 Cloning of LRP cassette into the donor pYLVS.

2.2.5.1.1.2 Cloning of *dapA* into donor vector SV

Cloning of *dapA* cassette into donor vector pYLSV was illustrated into Figure 2.4. Vector pTZ carrying *dapA* gene fused with chloroplastic transit peptide driven by phaseolin promoter was employed. The phasP-TP-dapA-phasT fragment was released from pTZ vector by *EcoRI* and *PstI* digestion. The released fragment was cloned into *EcoRI* and *PstI* digested donor vector pYLSV.

```
  1 atgttcacgg gaagtattgt cgcgattgtt actccgatgg atgaaaaagg taatgtctgt
 61 cgggctagct tgaaaaaact gattgattat catgtcgcca gcggtacttc ggcgatcgtt
121 tctgttggca ccactggcga gtccgctacc ttaaatcatg acgaacatgc tgatgtggtg
181 atgatgacgc tggatctggc tgatgggcgc attccggtaa ttgccgggac cggcgctaac
241 gctactgcgg aagccattag cctgacgcag cgcttcaatg acagtggtat cgtcggctgc
301 ctgacggtaa ccccttacta caatcgtccg tcgcaagaag gtttgtatca gcatttcaaa
361 gccatcgctg agcatactga cctgccgcaa attctgtata atgtgccgtc ccgtactggc
421 tgcgatctgc tcccggaaac ggtgggccgt ctggcgaaag taaaaaatat tatcggaatc
481 aaagaggcaa cagggaactt aacgcgtgta aaccagatca aagagctggt ttcagatgat
541 tttgttctgc tgagcggcga tgatgcgagc gcgctggact tcatgcaatt gggcggtcat
601 ggggttattt ccgttacgac taacgtcgca gcgcgtgata tggcccagat gtgcaaactg
661 gcagcagaag aacattttgc cgaggcacgc gttattaatc agcgtctgat gccattacac
721 aacaaactat ttgtcgaacc caatccaatc ccggtgaaat gggcatgtaa ggaactgggt
781 cttgtggcga ccgatacgct gcgcctgcca atgacaccaa tcaccgacag tggtcgtgag
841 acggtcagag cggcgcttaa gcatgccggt ttgctgtaa
```

Figure 2.3 The nucleotide sequence of *E. coli dapA* gene.
The underlined atg and taa represent the start and stop codon respectively.

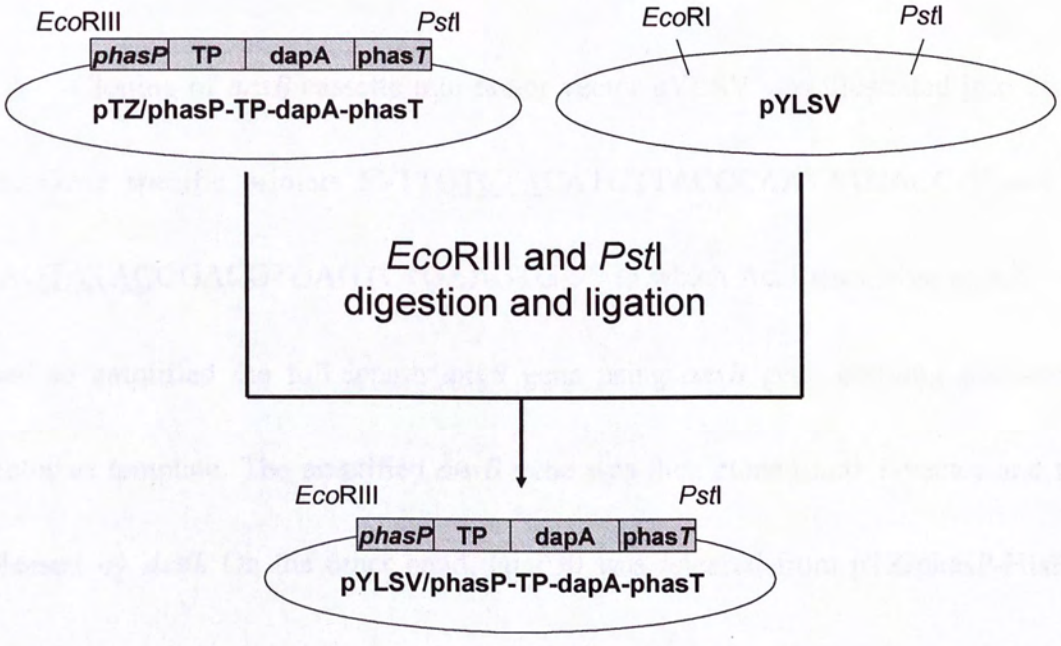


Figure 2.4 Cloning of dapA cassette into the donor pYLSV.

2.2.5.1.1.3 Cloning of *ansB* into donor vector VS

Cloning of *ansB* cassette into donor vector pYLSV was illustrated into Figure 2.6. Gene specific primers 5'-TTGTCTACATGTTACCCAATATCACC-3' and 5'-AAGTATACGGACGTGAGTCTGAAGTG-3', in which AccI sites were added, were used to amplified the full length *ansB* gene using *ansB* gene carrying pBluescript vector as template. The amplified *ansB* gene was then cloned into T-vector and then released by *AccI*. On the other hand, HisP30 was released from pTZ/phasP-HisP30-phasT by *AccI*. The *AccI* digested *ansB* gene was ligated to the pTZ carrying phaseolin promoter to form pTZ/phasP-ansB-phasT. *HindIII* digestion was then performed to release the phasP-ansB-phasT fragment from the resulting recombinant plasmid. The phasP-ansB-phasT fragment was ligated to *HindIII* digested donor vector pYLVS to form pYLVS/phasP-ansB-phasT.

```
   1 atggagtttt tcaaaaagac ggcacttgcc gcactggtta tgggttttag tggtgcagca
  61 ttggcattac ccaatatcac cattttagca accggcggga ccattgccgg tggtggtgac
 121 tccgcaacca aatctaacta cacagtgggt aaagttggcg tagaaaatct ggttaatgcg
 181 gtgccgcaac taaaagacat tgcgaacgtt aaaggcgagc aggtagtgaa tatcggctcc
 241 caggacatga acgataatgt ctggctgaca ctggcgaaaa aaattaacac cgactgcgat
 301 aagaccgacg gcttcgtcat tacccacggt accgacacga tggaagaaac tgcttacttc
 361 ctcgacctga cggtgaaatg cgacaaaccg gtggtgatgg tcggcgcaat gcgtccgtcc
 421 acgtctatga gcgcagacgg tccattcaac ctgtataacg cggtagtgac cgcagctgat
 481 aaagcctccg ccaaccgtgg cgtgctggta gtgatgaatg acaccgtgct tgatggccgt
 541 gacgtcacca aaaccaacac caccgacgta gcgaccttca agtctgttaa ctacggtcct
 601 ctgggttaca ttcacaacgg taagattgac taccagcgta ccccggcacg taagcatacc
 661 agcgacacgc cattcgatgt ctctaagctg aatgaactgc cgaaagtcgg cattgtttat
 721 aactacgcta acgcatccga tcttccggct aaagcactgg tagatgcggg ctatgatggc
 781 atcgttagcg ctggtgtggg taacggcaac ctgtataaat ctgtgttcga cacgctggcg
 841 accgccgcga aaaccggtac tgcagtcgtg cgttcttccc gcgtaccgac gggcgctacc
 901 actcaggatg ccgaagtgga tgatgcgaaa tacggcttcg tcgcctctgg cacgctgaac
 961 ccgcaaaaag cgcgcgttct gctgcaactg gctctgacgc aaaccaaaga tccgcagcag
1021 atccagcaga tcttcaatca gtactaa
```

Figure 2.5 The nucleotide sequence of *E. coli ansB* gene.
The underlined atg and taa represent the start and stop codon respectively.

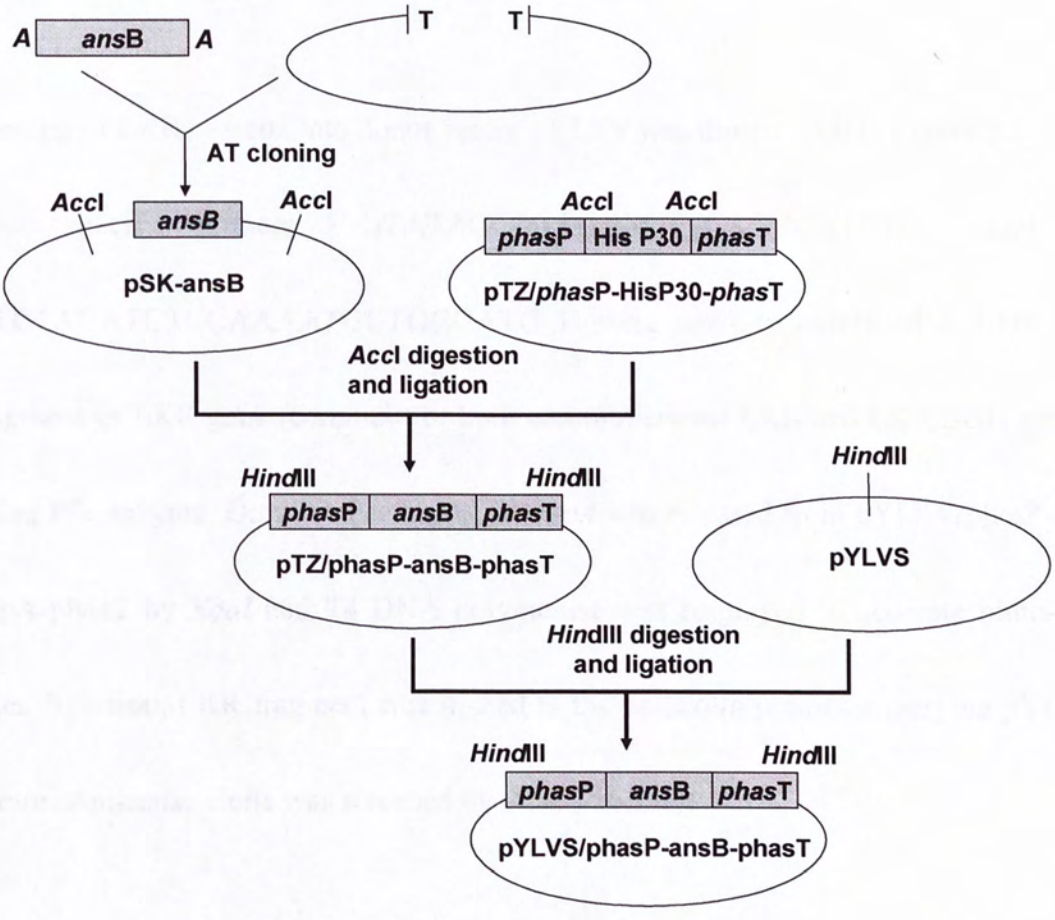


Figure 2.6 Cloning of ansB cassette into the donor pYLVS.

2.2.5.1.1.4 Cloning of antisense LKR fragment into donor vector SV

Cloning of LKR cassette into donor vector pYLSV was illustrated into Figure 2.8. Gene specific primers 5'-GTATACCATTGTGGTTCAGCCATCTG-3' and 5'-GTCTACATCTCCAAAATGCTGGGATG-3' were used to amplified a 1078 bps fragment of LKR gene (common for both monofunctional LKR and LKR/SDH genes) using Pfu enzyme. On the other hand, TP-*dapA* was released from pYLSV/phasP-TP-dapA-phasT by *XbaI* and T4 DNA polymerase was employed to generate blunt-end after digestion. LKR fragment was ligated to the phaseolin promoter carrying pYLVS vector. Antisense clone was screened by PCR screening.

cattgtggttcagccatctgctaagcgtatccatcatgatgccttgtatgaagatgttgggtgtgaaat

ttctgatgatttgtctgattgtgggcttatacttggaatcaaacaacctgagctagaaatgattcttcc

agagagagcatacgctttcttttcacatactcataaggcacagaaagagaacatgcctttgttggataa

aattctttctgagagagtgactttgtgtgattatgagctcattgttggggatcatgggaaacgattatt

ggcgtttggtaaatatgcaggcagagctggtcttgttgacttcttacacggacttggacagcgatatct

aagtctaggatactcaacacctttcctctcgctcggtgcatcgtatatgtattcctcattggctgctgc

aaaagccgctgtaatttctgttggtgaagaaattgcaagccagggactgccattaggaatctgccctct

tgtatttgtcttcaccggaacaggaaatgtttctctgggggcgcaagaaattttcaagcttcttcctca

cacttttgttgaaccaagcaaacttcctgaactatttgtaaaagacaaaggaattagtcaaaatgggat

ttcaacaaagcgagtctatcaagtatatggttgtattattaccagccaagacatggttgaacacaaaga

tccatcaaagtcattcgacaaagccgactattatgcacacccggaacattacaatccagttttccacga

aaagatatcgccatatacgtctgttcttgtaaactgtatgtactgggagaagagttttccctgtcttct

gagcacaaaacagcttcaagatttaacaaaaaaaggactcccactagtaggcatatgtgatataacttg

tgacatcggtggctccattgaatttgttaaccgagctactttaatcgattccccttttcttcaggtttaa

tccctcgaacaattcatactacgatgacatggatggggatggcgtactatgcatggctgttgacatttt

acccacagaatttgcaaaagaggcatcccagcattttggagat

Figure 2.7 The *LKR* fragment employed for antisense construct with the regions for primer annealing underlined.

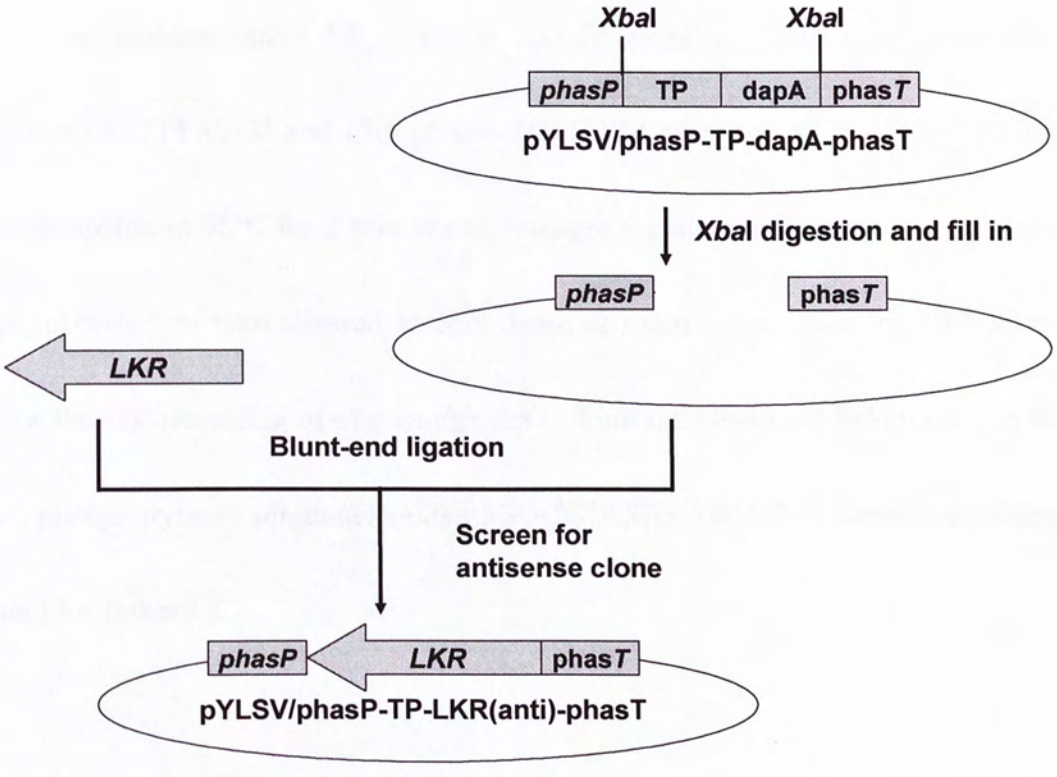


Figure 2.8 Cloning of antisense LKR cassette into the donor pYLSV.

2.2.5.1.2 Preparation of phosphorylated linkers

or making linker LS, 15μl of 200μM phosphorylated oligonucleotide 5'-GCGGCCGCTTAT-3' and 15ul of Tris-1/10EDTA were mixed in a 0.2ml PCR tube and incubated in 95°C for 2 minutes to denature the oligonucleotides to single-strand. The mixture was then allowed to cool down at room temperature for 30 minutes to allow the self-annealing of oligonucletides to form double-strand linker LS. For linker LV, phosphorylated oligonucleotides 5'-GCGGCCGCGCAC-3' were self-pairing as stated for linker LS.

2.2.5.1.3 Introduction of target genes to acceptor vector

The target gene cassettes subcloned into donor vector were introduced to the acceptor vector pYLTAC747H by cre-recombination (Figure 2.9) (Lin *et al.*, 2003). 100ng pYLTAC747H and 20ng *LRP* carrying donor vector pYLVS/*LRP* were co-electroporated into into Cre-recombinase expressing *E.Coli* strain NS3529. Colonies were selected on LB plates with kanamycin (20mg/l) and chloramphenicol (15mg/l). Each colony contains both cointegrated and independent parental plasmids. A mixture of these plasmids was isolated by miniprep, and was transformed into DH5α which do not express cre-recombinase. Colonies were selected on LB plates with kanamycin (20mg/l) and chloramphenicol (15mg/l). Resulting colonies contain either cointegrated plasmid or independent parental plasmids. Colony bearing cointegrated plasmid was

more transparent and was selected. Cointegrated plasmid was isolated and digested with I-*Sce*I to release the backbone of donor vector pYLVS. About 100μg of digested plasmid was religated by phosphorylated linker LS. The ligated product was transformed into DH5α by $CaCl_2$ transformation and colonies were selected on LB plate with kanamycin (25mg/l). The colonies were then tested for sensitivity on LB plate with chloramphenicol (25mg/l) to confirm the removal of backbone of donor vector. Religated plasmid pYLTAC747H/*LRP* was isolated for the second round of recombination.

pYLTAC747H/*LRP* and *dapA* carrying donor vector pYLSV/*dapA* were cointegrated by cre-recombination as stated above. Cointegrated plasmid was isolated and digested with PI-*Sce*I to release the backbone of donor vector pYLSV. About 100μg of digested plasmid was religated by phosphorylated linker LV. Religated plasmid pYLTAC747H/*LRP-dapA* was isolated for the third round of recombination.

pYLTAC747H/*LRP-dapA* and *ansB* carrying donor vector pYLVS/*ansB* were cointegrated by cre-recombination as stated above. Backbone of donor vector pYLVS were removed by I-*Sce*I digested and the plasmid was then religated by linker LS. The antisense LKR construct in donor vector pYLSV/*LKR* (antisense) was introduced to pYLTAC747H/*LRP-dapA-ansB* in the fourth round of recombination. Cointegrated

plasmid was isolated and digested with PI-*Sce*I to release the backbone of donor vector pYLSV. The acceptor vector carrying the four transgene cassettes was ready for plant transformation.

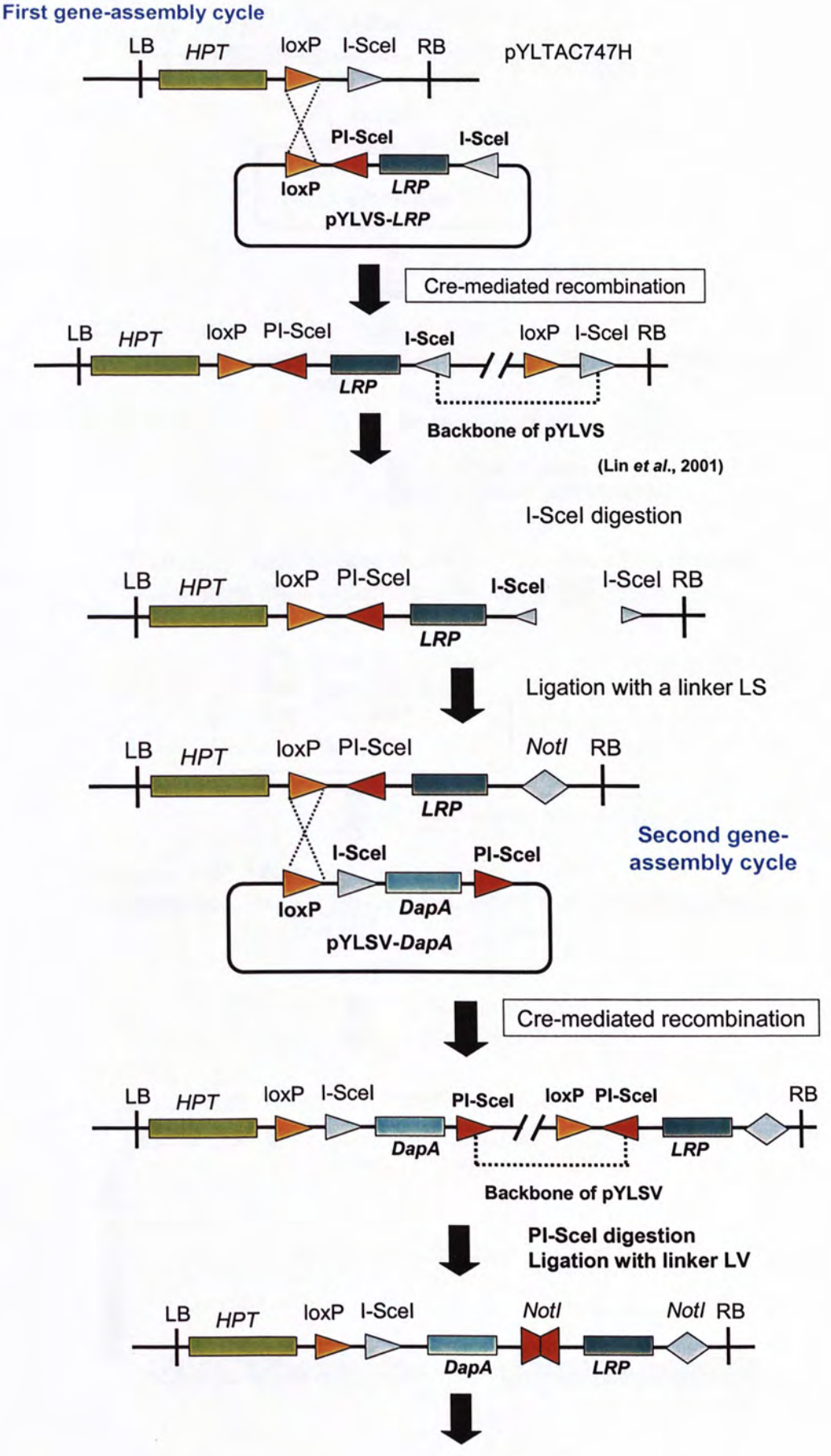


Figure 2.9 Construction of acceptor vector with four target gene cassettes.

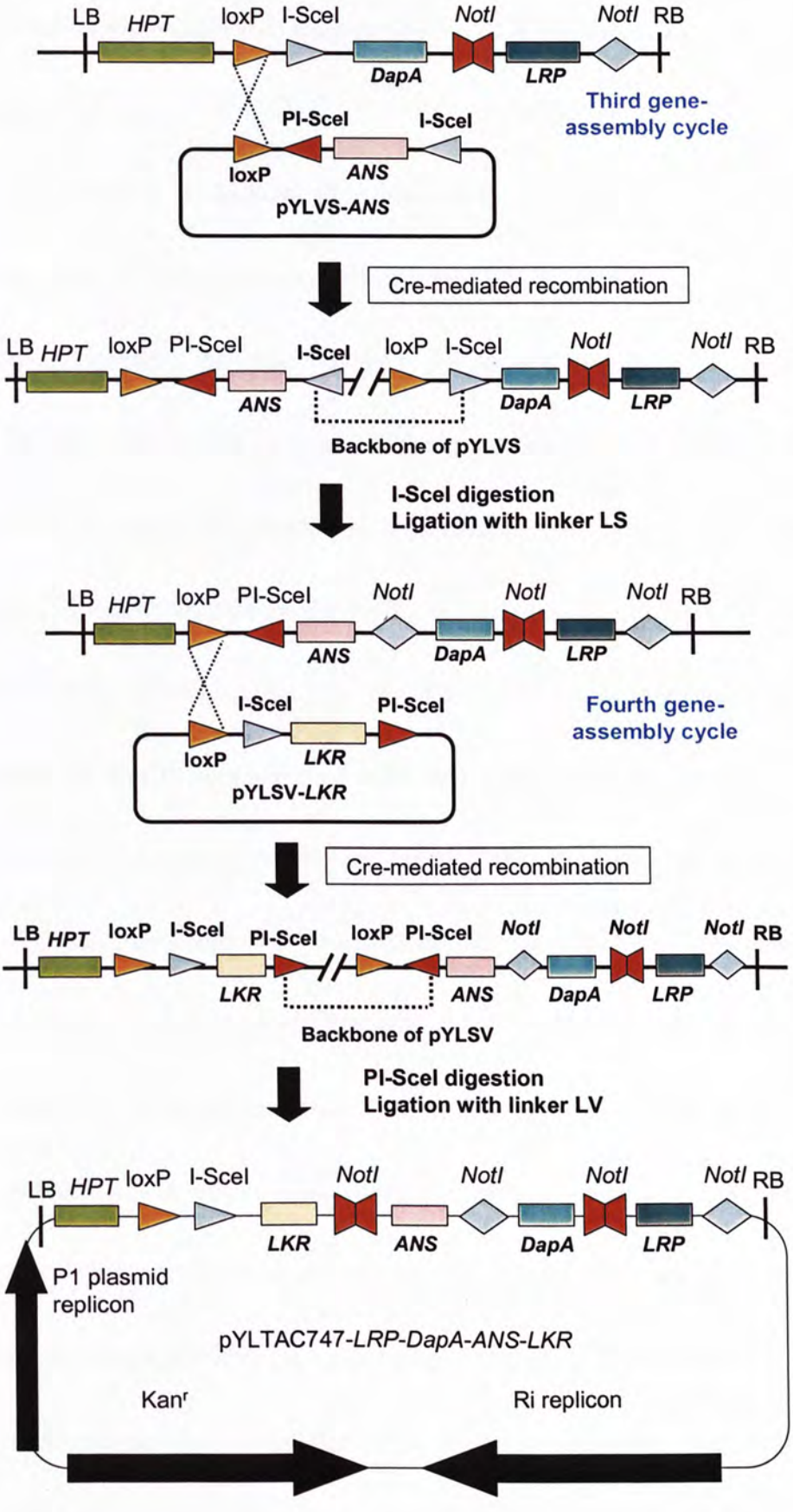


Figure 2.9 Construction of acceptor vector with four target gene cassettes (Continued).

2.2.5.2 *Agrobacterium*-mediated transformation of *A. thaliana* via vacuum infiltration

The seeds of *A. thaliana* were allowed to germinate and grow in soil in a growth chamber. When the primary inflorescence was 5-15cm tall and the secondary inflorescences appeared, the plant was ready for vacuum infiltration. All siliques and opened flowers were cut off prior to infiltration. A single colony of GV3101 with desired DNA construct was inoculated and cultured overnight in 10ml YEP with kanamycin (50 mg/L), rifampicin (50 mg/L) and gentamycin (25 mg/L) at 28°C with 300 rpm shaking. Subsequently, 500 ml YEP broth (10g/L tryptone, 10g/L yeast extract and 5g/L NaCl), supplemented with the three antibiotics, was inoculated with 1ml of the starter culture and grown overnight at 28°C with shaking at 300 rpm. When OD600 of the culture exceeds 2.0, the culture was spun down and resuspended in 1 liter of infiltration medium (2.2 g/L MS salts, 1x B5 vitamins, 50 g/L sucrose, 0.5 g/L MES, 0.044 uM benzylaminopurine and 0.02% Silwet$_{TM}$ L-77, pH 5.7). The resuspended culture was placed in a beaker and placed into a vacuum desiccator. Plants in the pots were inverted and immersed in the medium, followed by a vacuum of 400mmHg for 10 minutes after the appearance of bubbles in the medium. Vacuum was then released and the plants were allowed to recover. Plants were transferred back to the growth chamber and allowed to grow until brown dried seeds appear. This generation was regarded as T_0 and seeds of T_0 were harvested.

2.2.5.3 Screening of transformants

All the seeds of each individual transformant were surface sterilized. The surface sterilized seeds were spread onto petri plate containing MS medium supplemented with 20 mg/L hygromycin.

Chapter 3. Results

3.1 Characterization of transgenic lines with altered sink-source relationship

3.1.1 Amino acid analysis of mature seeds of transgenic lines

Col-0, 35S-*ASN1* over-expressers (362-D2-d6), phas-*PN2S* transgenic plant (C-6-3), phas-*LRP* transgenic plants (4-1-7), phas-*metL* transgenic plants (6-6-1), phas-*dapA*/phas-*LRP* transgenic plants (8-1-1) were grown and allow to shed seeds under identical conditions, as described in the 'Materials and Methods' section. The mature seeds were air-dried and the amino acid contents were analyzed. The absolute and relative amounts of acid-hydrolyzed amino acids were presented in Table 3.1 and 3.2.

Table 3.1 Absolute levels of amino acids in different transgenic lines after acid hydrolysis. The absolute levels of amino acids (after acid hydrolysis) of the wild-type Col-0, 35S-*ASN1* over-expressers (362-D2-d6), phas-*PN2S* transgenic plant (C-6-3), phas-*LRP* transgenic plants (4-1-7), phas-*metL* transgenic plants (6-6-1), and phas-*dapA*/phas-*LRP* transgenic plants (8-1-1) were compared. The absolute levels of amino acid in different lines were subject to one-way ANOVA analysis. * indicates *p*-values less than 0.05 when compared to the wild type Col-0. Standard deviation is shown in parentheses. Asp Family Ess stands for aspartate family essential amino acid, comprising of Lys, Met, Thr and Ile.

Lines	Col-0	362-D2-d6	C-6-3	4-1-7	6-6-1	8-1-1
Asx (Asn+Asp)	12.73 (0.71)	16.05* (0.35)	13.14 (1.09)	12.50 (0.14)	12.35 (1.49)	11.33 (0.75)
Ser	7.97 (0.32)	9.10 (0.00)	8.50 (0.76)	8.05 (0.21)	7.78 (0.69)	6.97 (0.96)
Glx	28.60 (0.79)	33.35* (0.21)	29.58 (2.01)	27.65 (2.19)	28.00 (3.71)	25.13 (1.40)
Gly	11.13 (0.25)	12.95 (0.07)	10.02 (0.73)	12.50 (3.96)	10.63 (0.98)	10.57 (0.85)
His	4.03 (0.23)	5.10* (0.14)	4.00 (0.52)	3.90 (0.28)	3.90 (0.75)	4.07 (0.32)
Arg	11.77 (0.31)	13.25 (0.35)	12.50 (0.73)	11.35 (1.20)	11.48 (1.24)	10.20* (0.70)
Thr	6.93 (0.12)	7.50 (0.14)	6.72 (0.32)	7.10 (0.71)	7.08 (0.68)	6.73 (0.45)
Ala	7.47 (0.21)	8.50* (0.57)	7.42 (0.43)	7.25 (0.21)	7.25 (0.58)	6.80 (0.61)
Pro	9.27 (0.21)	10.25 (0.07)	8.68 (0.48)	8.80 (0.99)	8.58 (0.87)	8.43 (0.58)
Tyr	4.37 (0.21)	5.40* (0.28)	4.52 (0.29)	4.25 (0.07)	4.68 (0.64)	4.27 (0.38)
Val	8.47 (0.51)	9.45 (0.35)	7.48* (0.58)	7.80 (0.42)	8.08 (0.75)	7.13* (0.23)
Met	2.43 (0.12)	2.80 (0.42)	3.88* (0.26)	2.55 (0.07)	2.78 (0.30)	1.83* (0.12)
Lys	10.77 (0.06)	11.70 (0.42)	9.94 (0.70)	10.45 (0.21)	10.68 (0.63)	10.53 (0.76)
Ile	7.33 (0.06)	8.05 (0.07)	6.50* (0.44)	6.60 (0.42)	6.98 (0.59)	6.40* (0.40)
Leu	11.40 (0.82)	12.55 (0.07)	10.64 (0.55)	10.05 (0.78)	10.50 (1.04)	9.33* (0.47)
Phe	7.67 (0.51)	8.55 (0.21)	6.76 (0.53)	7.00 (0.28)	7.30 (0.91)	6.67 (0.40)
Protein	130.60 (2.78)	149.75* (0.21)	129.04 (8.37)	126.40 (9.76)	127.00 (13.13)	116.90 (6.10)
Asp Family Ess (Lys+Met+Thr+Ile)	27.47 (0.12)	30.05 (0.78)	27.04 (1.64)	26.70 (1.41)	27.50 (2.15)	25.50 (1.42)

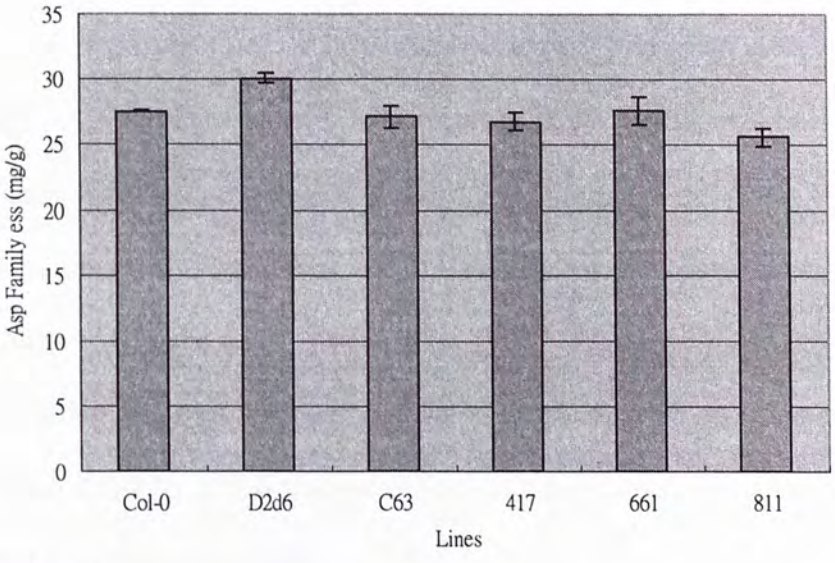
Table 3.2 Relative level of amino acids in different transgenic lines. The relative levels of amino acids (after acid hydrolysis) of the wild-type Col-0, 35S-*ASN1* over-expressers (362-D2-d6), phas-*PN2S* transgenic plant (C-6-3), phas-*LRP* transgenic plants (4-1-7), phas-*metL* transgenic plants (6-6-1), and phas-*dapA*/phas-*LRP* transgenic plants (8-1-1) were compared. * indicates *p*-values less than 0.05 when compared (by one-way ANOVA) to the wild type Col-0. Standard deviation is shown in parentheses. Asp Family Ess stands for aspartate family essential amino acid, comprising of Lys, Met, Thr and Ile.

Lines	Col-0	362-D2-d6	C-6-3	4-1-7	6-6-1	8-1-1
Asx (Asn+Asp)	7.93 (0.32)	8.75* (0.21)	8.36 (0.25)	7.95 (0.64)	7.93 (0.30)	7.90 (0.10)
Ser	6.27 (0.15)	6.30 (0.00)	6.84 (0.34)	6.55 (0.78)	6.33 (0.30)	6.10 (0.62)
Glx	16.13 (0.40)	16.40 (0.14)	17.02* (0.41)	15.95 (0.21)	16.25 (0.65)	15.77 (0.55)
Gly	12.30 (0.10)	12.55 (0.07)	11.32* (0.29)	13.95 (3.18)	12.18 (0.24)	12.97 (0.40)
His	2.20 (0.17)	2.40 (0.00)	2.16 (0.15)	2.10 (0.00)	2.15 (0.31)	2.40 (0.10)
Arg	5.60 (0.10)	5.50 (0.14)	6.10* (0.21)	5.55 (0.07)	5.65 (0.06)	5.40 (0.10)
Thr	4.83 (0.15)	4.55* (0.07)	4.76 (0.15)	5.05 (0.07)	5.10* (0.08)	5.20* (0.10)
Ala	6.93 (0.15)	6.90 (0.42)	7.04 (0.13)	6.95 (0.49)	7.00 (0.26)	7.03 (0.31)
Pro	6.70 (0.26)	6.45 (0.07)	6.38 (0.18)	6.50 (0.14)	6.38 (0.22)	6.80 (0.60)
Tyr	2.00 (0.10)	2.15 (0.07)	2.08 (0.04)	2.00 (0.14)	2.23 (0.13)	2.20 (0.10)
Val	6.03 (0.21)	5.85 (0.21)	5.40* (0.12)	5.65* (0.21)	5.90 (0.08)	5.63* (0.15)
Met	1.37 (0.06)	1.35 (0.21)	2.18* (0.04)	1.45 (0.07)	1.60* (0.08)	1.13* (0.12)
Lys	6.10 (0.17)	5.80 (0.14)	5.76 (0.15)	6.10 (0.42)	6.28 (0.26)	6.63* (0.40)
Ile	4.67 (0.06)	4.45 (0.07)	4.22* (0.08)	4.30* (0.14)	4.53 (0.05)	4.53 (0.32)
Leu	7.20 (0.44)	6.90 (0.00)	6.86 (0.18)	6.50* (0.14)	6.83* (0.13)	6.57* (0.12)
Phe	3.83 (0.21)	3.75 (0.07)	3.48* (0.08)	3.55* (0.21)	3.80 (0.08)	3.70 (0.00)
Asp Family Ess (Lys+Met+Thr+Ile)	16.97 (0.38)	16.15* (0.35)	16.92 (0.13)	16.90 (0.57)	17.50 (0.36)	17.50 (0.78)

3.1.1.1 Aspartate family amino acids levels remain steady in seeds of transgenic plants

Aspartate family essential amino acids, especially Lys and Met, are major targets for improvement of seed proteins. Thus, the absolute and relative levels of aspartate family essential amino acids (Lys+Met+Thr+Ile) in mature seeds of different transgenic lines were compared (Fig 3.1) For all transgenic lines studied, the absolute amount of aspartate family amino acids did not show significant difference when compared to the wild-type Col-0. The relative levels of aspartate family essential amino acids of the transgenic lines also showed no significant difference to wild-type Col-0. There was a slight decrease in the relative amount of aspartate family essential amino acids in 35S-*ASN1* line. In general, all transgenic constructs cannot successfully improve the level of aspartate family essential amino acids in seeds.

A. Absolute levels



B. Relative Levels

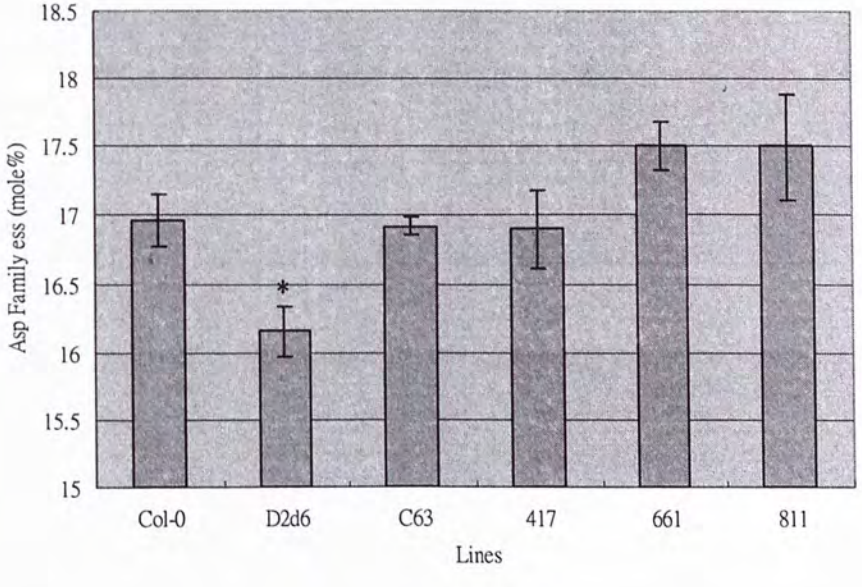
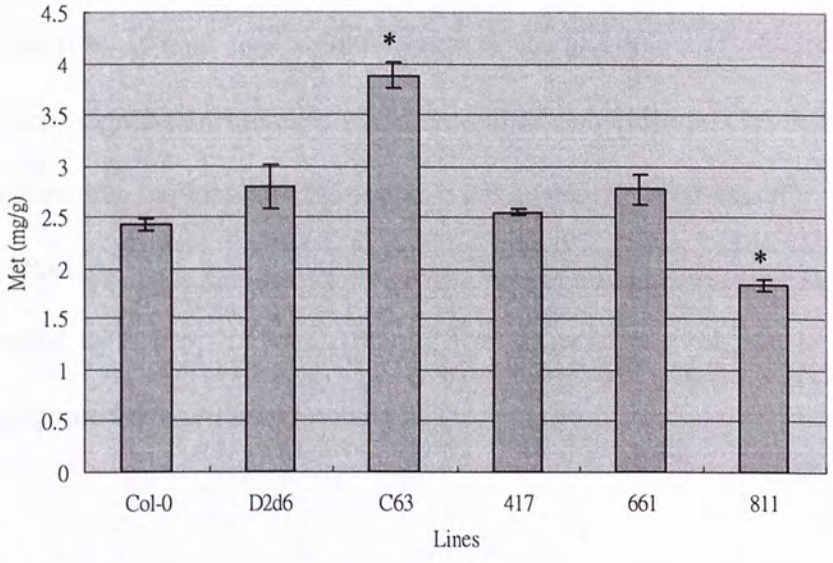


Figure 3.1 Absolute and relative levels of aspartate family essential amino acids in different transgenic lines. The absolute levels (Panel A) and relative levels (Panel B) of acid-hydrolyzed aspartate family essential amino acids (Lys+Met+Thr+Ile) of wild-type (Col-0), 35S-*ASN1* over-expressers (362-D2-d6), phas-*PN2S* transgenic plant (C-6-3), phas-*LRP* transgenic plants (4-1-7), phas-*metL* transgenic plants (6-6-1), and phas-*dapA*/phas-*LRP* transgenic plants (8-1-1) were compared. * indicates *p*-values less than 0.05 when compared (by one-way ANOVA) to the wild type Col-0. Each bar represents an average of two or three samples. Error bars: standard deviations.

3.1.1.2 Increase in seed Met content in Met-rich protein expressing transgenic plants

The level of Met in seeds of *A. thaliana* was very low when compared to other amino acids. When comparing with wild-type Col-0, the transgenic plants expressing a seed-specific Met-rich protein PN2S showed significant increases in both absolute and relative amount of Met (Figure 3.2). A slight drop in relative amount of lysine was also observed in the seeds of the phas-*PN2S* transgenic plant. The transgenic plants expressing seed-specific *metL* gene also showed a slight increase in the absolute amount of Met. Other transgenic lines did not show significant change in the absolute and relative levels of Met

A. Absolute levels



B. Relative levels

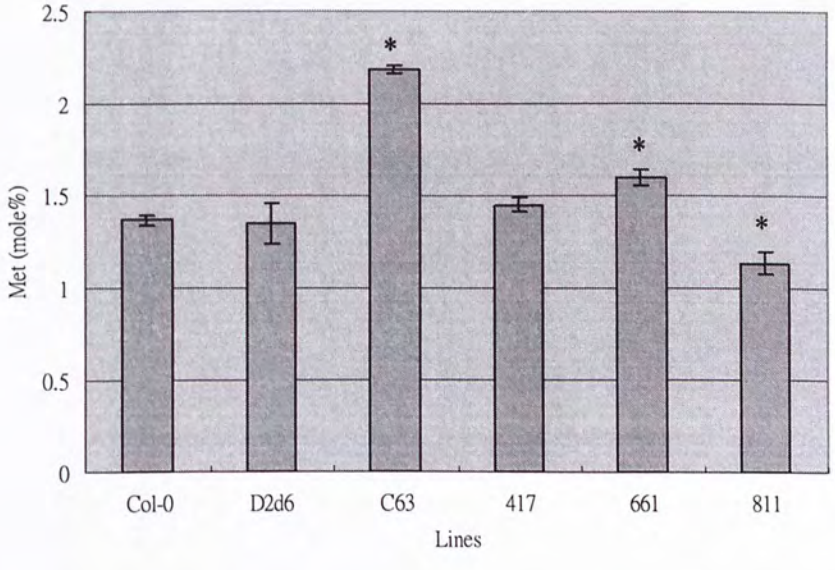
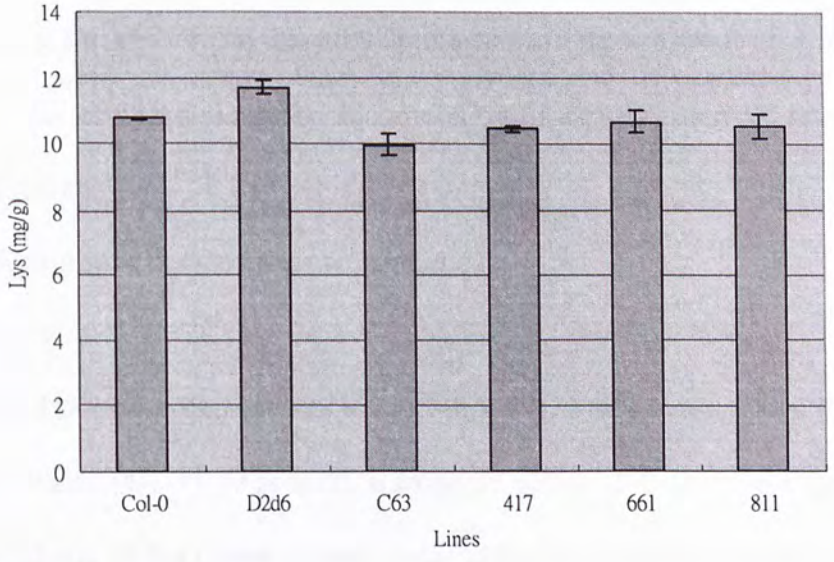


Figure 3.2 Absolute and relative levels of Met in different transgenic lines. The absolute levels (Panel A) and relative levels (Panel B) of Met (after acid hydrolysis) of wild-type (Col-0), 35S-*ASN1* over-expressers (362-D2-d6), phas-*PN2S* transgenic plant (C-6-3), phas-*LRP* transgenic plants (4-1-7), phas-*metL* transgenic plants (6-6-1), and phas-*dapA*/phas-*LRP* transgenic plants (8-1-1) were compared. * indicates *p*-values less than 0.05 when compared (by one-way ANOVA)to the wild type Col-0. Each bar represents an average of two or three samples. Error bars: standard deviations.

3.1.1.3 Increase in seed Lys content in phas-*dapA*/phas-*LRP* transgenic plants

Although previous study showed that the phas-*LRP* transgenic plants might account for 10% of total seed soluble proteins, the absolute and relative levels of Lys did not show significant increase when compared to wild-type Col-0 (Figure 3.3). In transgenic plants expressing *dapA* and *LRP* genes in seed-specific promoter, the relative level of Lys was enhanced slightly. However, that increase in Lys was accompanied by a drop in Met content. The absolute and relative levels of Lys in other transgenic lines remained constant.

A. Absolute level



B. Relative level

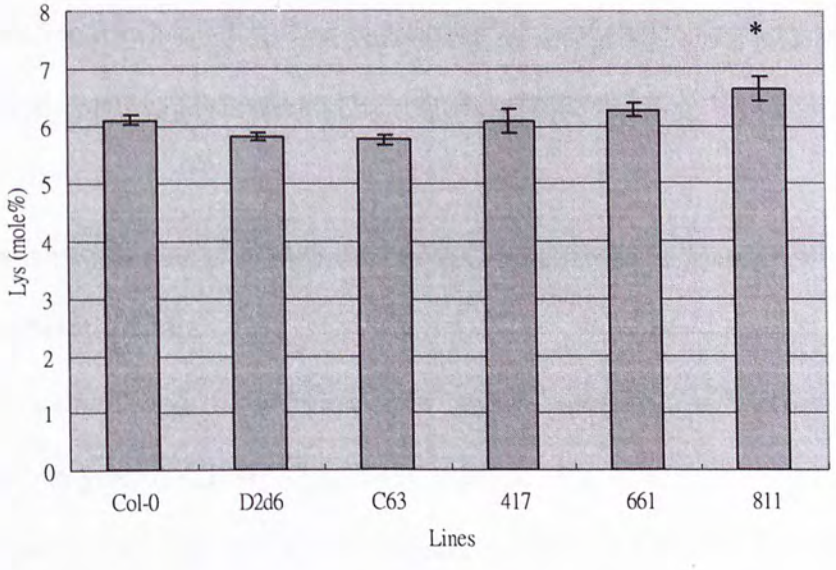


Figure 3.3 Absolute and relative levels of Lys in different transgenic lines. The absolute levels (Panel A) and relative levels (Panel B) of Lys (after acid hydrolysis) of wild-type (Col-0), 35S-*ASN1* over-expressers (362-D2-d6), phas-*PN2S* transgenic plant (C-6-3), phas-*LRP* transgenic plants (4-1-7), phas-*metL* transgenic plants (6-6-1), and phas-*dapA*/phas-*LRP* transgenic plants (8-1-1) were compared. * indicates *p*-values less than 0.05 when compared (by one-way ANOVA) to the wild type Col-0. Each bar represents an average of two or three samples. Error bars: standard deviations.

3.1.2 Gene expression study of transgenic line

Amino acid analysis showed that the transgenic constructs studied did not enhance the aspartate-derived essential amino acids in mature seeds of *A. thaliana*. To investigate the key obstacle in the accumulation of aspartate-derived amino acids in seeds, the expressions of key regulatory enzymes of aspartate family amino acid pathway during seed development were studied.

Col-0, *ASN1* over-expressers (362-D2-d6), *PN2S* transgenic plants (C-6-3), *LRP* transgenic plants (4-1-7), phas-*metL* transgenic plants (6-6-1), phas-*dapA*/phas-*LRP* transgenic plants (8-1-1) were grown under identical conditions, as described in the Section 2.2.2. The siliques at different developmental stages were collected as described in Section 2.2.3.2.1. The expression of genes encoding key enzymes in aspartate family pathway was studied by real-time PCR.

3.1.2.1 Down-regulation of *akthr1* and *akthr2* in transgenic plants with altered N sink-source relationship

The *akthr1* and *akthr2* are two genes encoding a threonine-sensitive bifunctional enzyme AK/HSD. AK/HSD catalyzes the first committed step in the aspartate family pathway and is an important control of the metabolic flux into aspartate family pathway. The expression levels of *akthr1* in developing siliques of different constructs were compared. In siliques of 10 DAF, there is a significant decrease in *akthr1* expression in *PN2S* transgenic plants (C-6-3), *LRP* transgenic plants (4-1-7), phas/*metL* transgenic plants (6-6-1), *dapA*/*LRP* transgenic plants (8-1-1) when compared to wild-type the Col-0 (Figure 3.4A).

The expression of *akthr2* showed a similar trend with *akthr1* in 10 DAF siliques. There was also a down regulation of *akthr2* expression in transgenic lines C-6-3, 4-1-7, 6-6-1 and 8-1-1 (Figure 3.4B).

Previous research found that the promoter of *akthr1* contains GCN4-like motif and opaque2-like motif, while the promoter of *akthr2* contains two GCN4-like motifs (Rognes *et al.*, 2003). Thus, it is possible that *akthr1* and *akthr2* are regulated by general amino acid control (GAAC) in *A. thaliana*.

3.1.2.2 Down regulation of *GCN2* in transgenic plants with altered N sink-source relationship

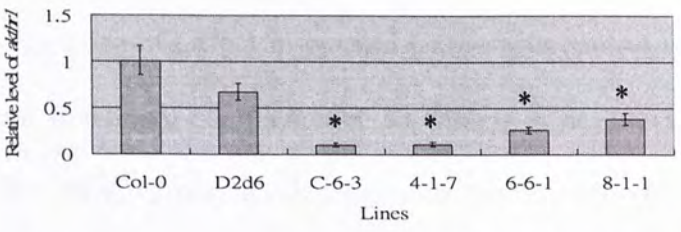
To investigate the correlation of *akthr1* and *akthr2* with GAAC, the expression of *GCN2* (a key component of GAAC) was studied. The expression of *GCN2* was also down-regulated in transgenic lines C-6-3, 4-1-7, 6-6-1 and 8-1-1 (Figure 3.4C). The result suggested a correlation between GCN2 and the expression of *akthr1* and *akthr2*, and the two genes might be targets of GAAC.

3.1.2.4 Expression study of other genes in aspartate family pathway

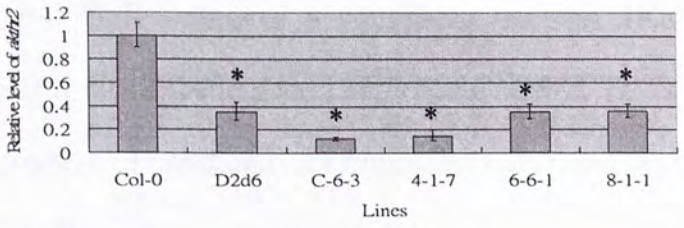
To investigate whether the down regulation is a general trend for all aspartate family biosynthetic genes, the expression of *dhps2* and *CGS* were studied. The *dhps2* encodes for dihydrodipicolinate synthase, the key regulatory enzyme for lysine biosynthesis. By real-time PCR, the expression levels of *dhps2* were studied and the *dhps2* levels in different transgenic constructs did not show significant difference when compared to the wild-type Col-0 (Figure 3.5D).

CGS is a key regulatory enzyme in methionine biosynthesis. Result of real-time PCR showed that the *CGS* expression in the transgenic plants C-6-3, 4-1-7 and 8-1-1 was similar to that of wild-type Col-0 (Figure 3.5E). There is a slight drop in *CGS* level in transgenic plant expressing *E. coli metL* (6-6-1). The results suggested that the down regulation is specific for *akthr1* and *akthr2* but not a general trend for all aspartate family amino acid biosynthetic genes.

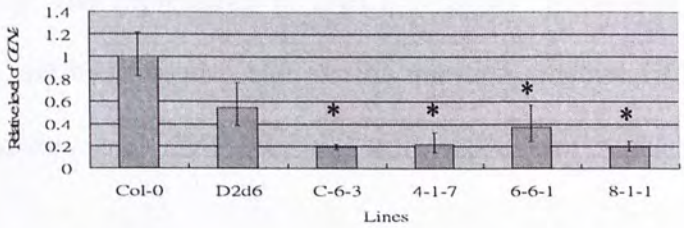
A. *akthr1*



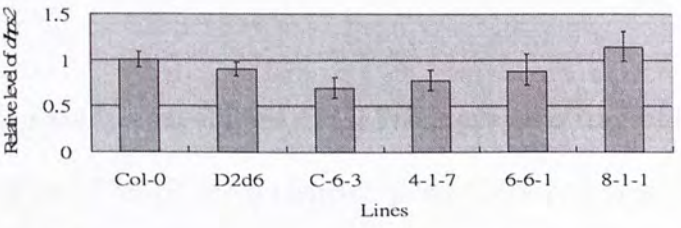
B. *akthr2*



C. *GCN2*



D. *dhps2*



E. *CGS*

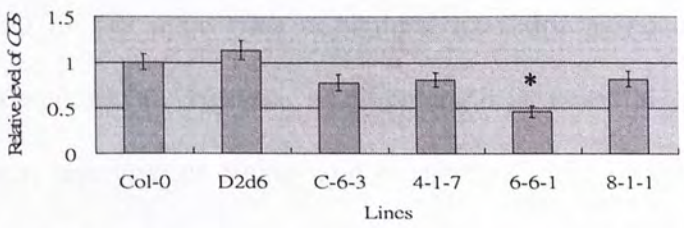


Figure 3.4. Change of gene expression levels of transgenic line with altered N sink-source relationship during seed development.

Panel A to E: *akthr1*, *akthr2*, *GCN2*, *dhps2*, and *CGS*, respectively. Siliques of 10 days after flowering (DAF) were collected for RNA extraction and cDNA synthesis as described in Materials and Methods. Real-time PCR was performed as described in Materials and Methods. All reactions were repeated independently for at least three times to obtain consistent results. Data was normalized using actin. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The changes in mRNA expression level were tested by an independent *t*-test, * indicates *p*-values less than 0.05 when compared to wild type Col-0. Each bar represents an average of two or three repeats. Error bars: standard deviations.

3.2 Characterization of *GCN2* overexpressing line

Although the homolog of GCN2 in general amino acid control was identified in *A. thaliana* by yeast functional complementation (Zhang *et al.*, 2003), there is no direct evidence that the *AtGCN2* play a role in general amino acid control *in planta*. The results of real-time PCR suggested a correlation between *AtGCN2* and the expression of amino acid biosynthetic genes, *akthr1* and *akthr2*. To further confirm this correlation, transgenic *A. thaliana* constitutively overexpressing *GCN2* was employed (Ma, 2005). The expressions of aspartate family amino acid biosynthetic genes were studied, and this can also address the question whether *GCN2* regulates the expression of *akthr1* and *akthr2* or the three genes are regulated by same mechanism.

3.2.1 Gene expression study of seedlings of GCN2 overexpressing plants

Wild-type Col-0 and 35S-*GCN2* transgenic plant (GCN2A) were grown in MS plates and transferred to MS plate with or without azaserine as stated in Section 2.2.4.1. Azaserine is a glutamine analogue, as glutamine is an essential nitrogen donor for the transamination reactions in amino acid biosynthesis, the action of azaserine will cause a global deficiency in amino acid pools. Azaserine treatment will generate an amino acid deprived condition, which is the condition for activation of GCN2 in yeast system. 14 day-old seedlings were used for expression analysis by real-time PCR.

3.2.1.1 Increased *GCN2* expression by azaserine treatment

From real-time PCR, the expression of *GCN2* is strongly induced in the 35S-*GCN2* line when compared to wild-type Col-0 in both normal and amino acid

deprived condition (Figure 3.5A). It is also found that the expressions of *GCN2* in both wild-type Col-0 and 35S-*GCN2* were induced in amino acid deprived condition (by azaserine treatment).

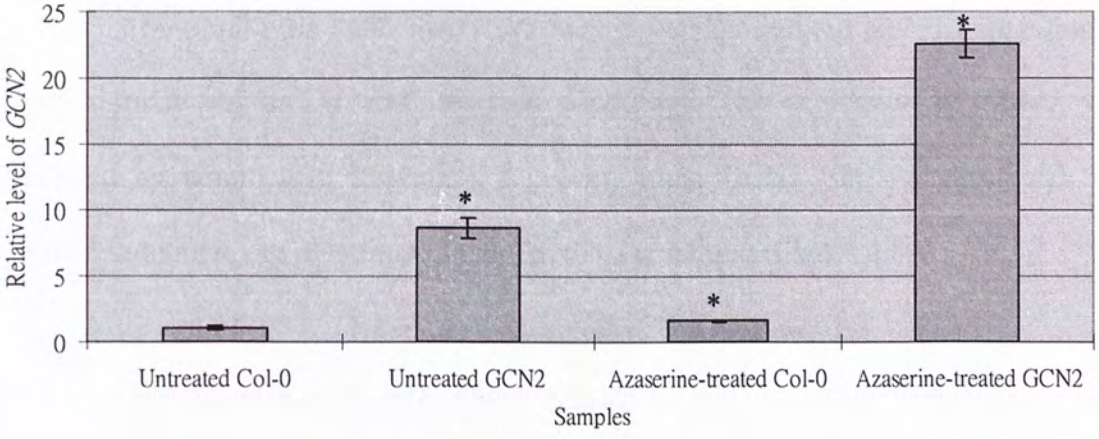


Figure 3.5. Change of *GCN2* expression levels of *GCN2* overexpressing line in normal and amino acid deprived conditions. The wild-type Col-0 and *GCN2* overexpressing line were grown in MS plates for 11 days and transferred to MS plates with or without azaserine for treatment of 3 days as described in Materials and Methods. Real-time PCR was performed and gene expression level was calculated as described in Materials and Methods. * indicates *p*-values less than 0.05 when compared (by *t*-test) to the untreated wild type Col-0. Each bar represents an average of two or three repeats. Error bars: standard deviations.

3.2.1.2 Increased *akthr1* and *akthr2* expression in *GCN2* overexpressing plants

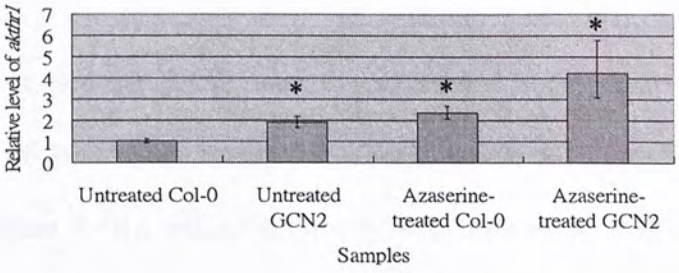
From real-time PCR, the *GCN2* overexpression induced *akthr1* expression in both normal and amino acid deprived conditions. The expression of *akthr1* was induced by amino acid starvation, however, when GCN2 was overexpressed, the *akthr1* induction was also found in non-starved condition (Figure 3.6A).

The expression of *akthr2* was similar to that of *akthr1*. Both amino acid starvation and GCN2 overexpression induced the expression of *akthr2* (Figure 3.6C). The induction of *akthr1* and *akthr2* in 35S-*GCN2* line suggested the regulation of the expression of these two genes by GCN2.

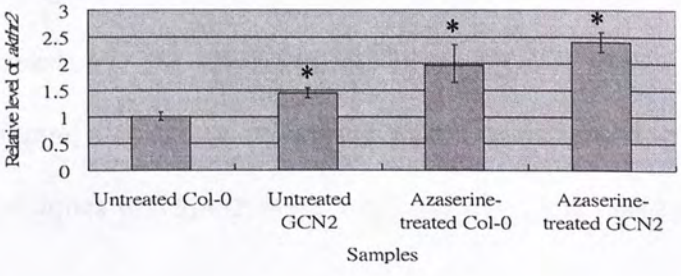
3.2.1.3 Expression study of other genes in aspartate family pathway

To investigate the effect of GCN2 on the aspartate family pathway, the expression of *dhps2* and *CGS* were also studied. It was found that GCN2 overexpression did not altered the expressions of the two genes (Figure 3.6C and Figure 3.6D). This indicated that the action of GCN2 is gene specific.

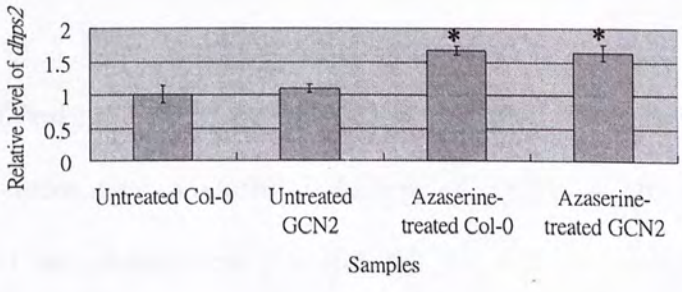
A. *akthr1*



B. *akthr2*



C. *dhps2*



D. *CGS*

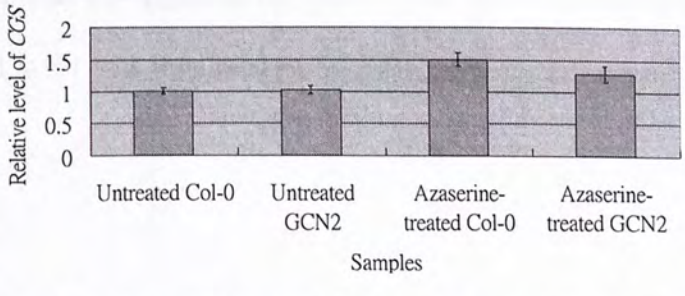


Figure 3.6. Change of gene expression levels of *GCN2* overexpressing line in normal and amino acid deprived conditions.

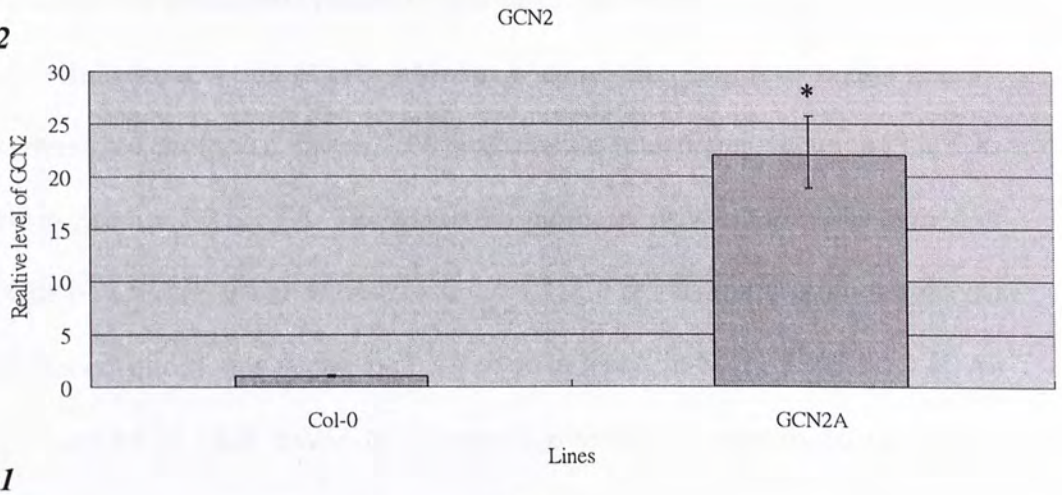
Panel A to D: *akthr1*, *akthr2*, *dhps2*, and *CGS* The wild-type Col-0 and *GCN2* overexpressing line were grown in MS plates for 11 days and transferred to MS plates with or without azaserine for treatment of 3 days as described in Materials and Methods. Real-time PCR was performed and gene expression level was calculated as described in Materials and Methods. * indicates *p*-values less than 0.05, when compared (by *t*-test) to the untreated wild type Col-0. Each bar represents an average of two or three repeats. Error bars: standard deviations.

3.2.2 Gene expression study of GCN2 overexpressing plants during seed development

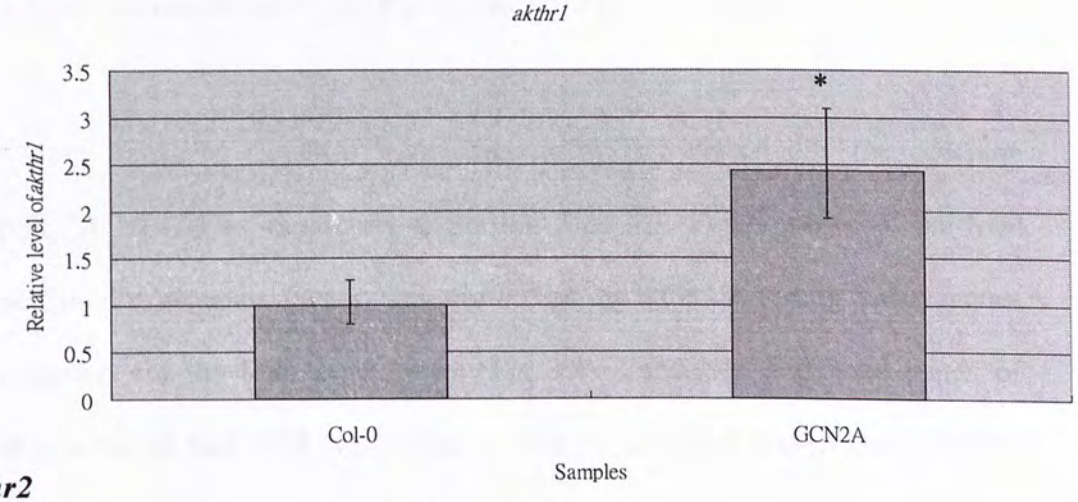
As mentioned in Section 3.1.2, *akthr1* and *akthr2* were down-regulated in developing siliques of 10DAF in transgenic plants C-6-3, 4-1-7, 6-6-1 and 8-1-1 (Figure 3.4A and Figure 3.4B), while this down-regulation correlated with a down-regulation of *GCN2* (Figure 3.4C). To investigate if the down-regulation of *akthr1* and *akthr2* was caused by the down-regulation of *GCN2*, *akthr1* and *akthr2* expressions in developing siliques of transgenic plants overexpressing *GCN2* were studied. Developing siliques of 10DAF were employed for gene expression analysis by real-time PCR.

The result of real-time PCR showed that in the developing siliques of 35S-*GCN2* line, where there was a strong induction of *GCN2* (Figure 3.7A), the expressions of *akthr1* and *akthr2* were also induced (Figure 3.7B and Figure 3.7C). This suggested that the down-regulation of *akthr1* and *akthr2* in transgenic plants C-6-3, 4-1-7, 6-6-1 and 8-1-1 was regulated by GCN2.

A. *GCN2*



B. *akthr1*



C. *akthr2*

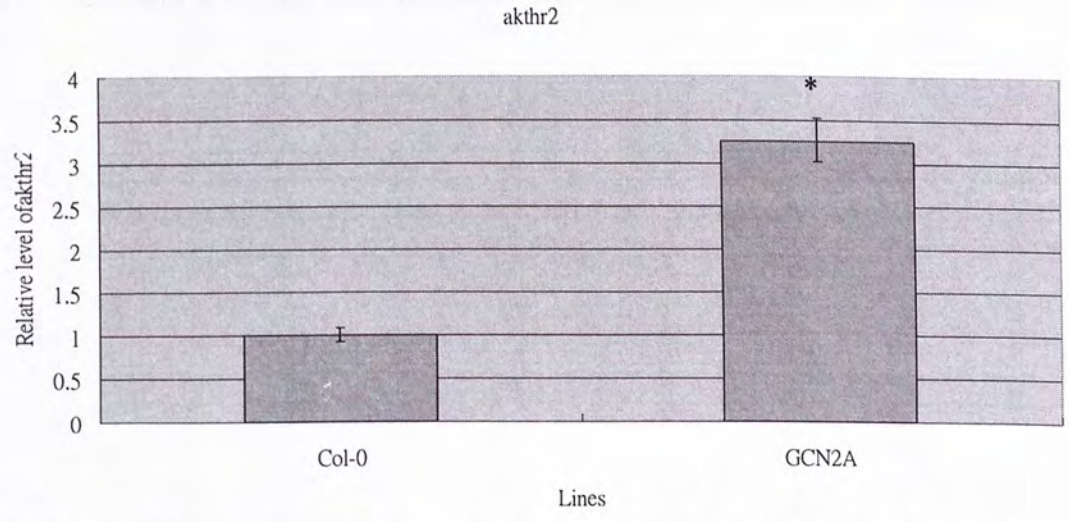


Figure 3.7 Change of gene expression levels of *GCN2* overexpressing line during seed development.

Panel A to C: *GCN2*, *akthr1*, *akthr2*, respectively. Siliques of 10 day after flowering (DAF) were collected for real-time PCR. Real-time PCR was preformed and gene expression level was calculated as described in Figure 3.4. * indicates *p*-values less than 0.05, when compared (by *t*-test) to the untreated wild type Col-0. Each bar represents an average of two or three repeats. Error bars: standard deviations

3.3 Construction of transgenic plants by multigene assembly system

3.3.1 Successful construction of recombinant plasmid carrying four target genes

The phaseolin promoter driven *LRP* was cloned into donor vector pYLVS as mentioned in Section 2.2.5.1.1.1. The phaseolin promoter driven *dapA* was cloned in donor vector pYLSV as stated in Section 2.2.5.1.1.2. The phaseolin promoter driven *ansB* was cloned into donor vector pYLVS as mentioned in Section 2.2.5.1.1.3. An antisense fragment of *LKR* driven by phaseolin promoter as mentioned in Section 2.2.5.1.1.4 and the orientation of *LKR* was confirmed by DNA sequencing.

The four transgene cassettes were subsequently introduced into the acceptor vector pYLTAC 747H as mentioned in Section 2.2.5.1.3. The presence of the four transgenes in the acceptor vector was confirmed by PCR screening using gene-specific primers for the four target genes (Fig 3.8). Candidate 2 showed bands of expected size for all four PCR were selected. The recombinant plasmid carrying the *LRP*, *dapA*, *ansB* and antisense *LKR* cassettes was successfully constructed (Figure 3.9).

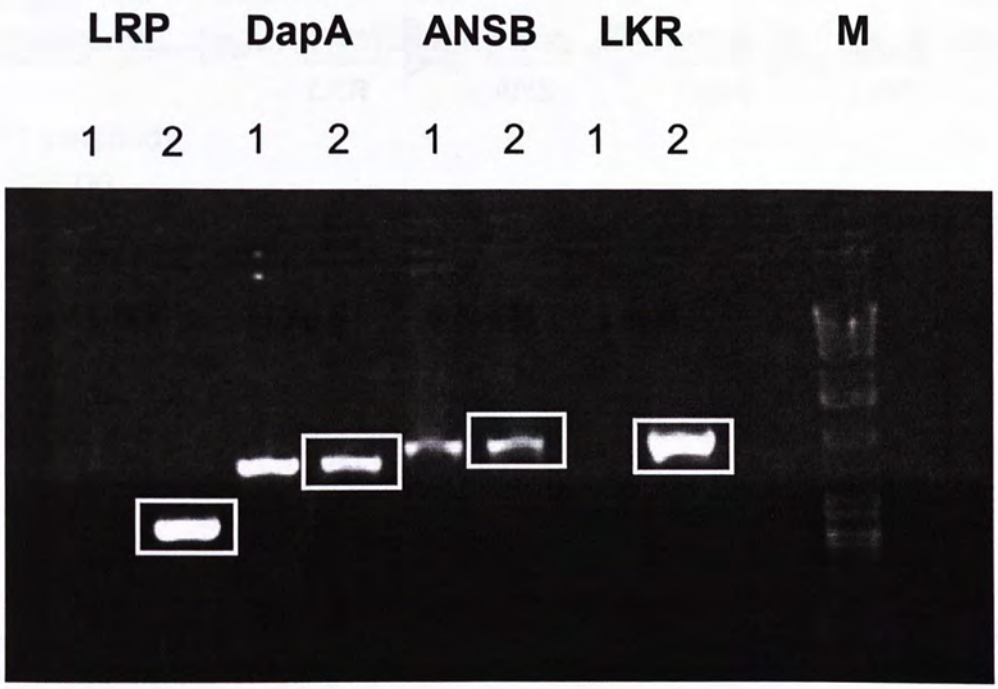


Figure 3.8 PCR screening of the four transgenes in recombinant plasmids.
The four target gene cassettes (*LRP*, *dapA*, *ansB*, and antisense *LKR*) were introduced to the acceptor vector pYLTAC747H by four rounds of cre-recombination as described in Materials and Methods. PCR screening of the recombinant plasmid was performed with gene specific primers for *LRP*, *dapA*, *ansB*, and *LKR* genes. The PCR products were run on 1% agarose gel stained with 10ng/ul ethidium bromide and photo was taken upon UV illumination. Lane 1 and 2: *LRP* PCR; lane 3 and 4: *dapA* PCR; lane 5 and 6: *ansB* PCR; lane 7 and 8: *LKR* PCR; lane 1,3,5 and 7: candidate 1; lane 2,4,6 and8: candidate 2; M: 1kb molecular weight markers

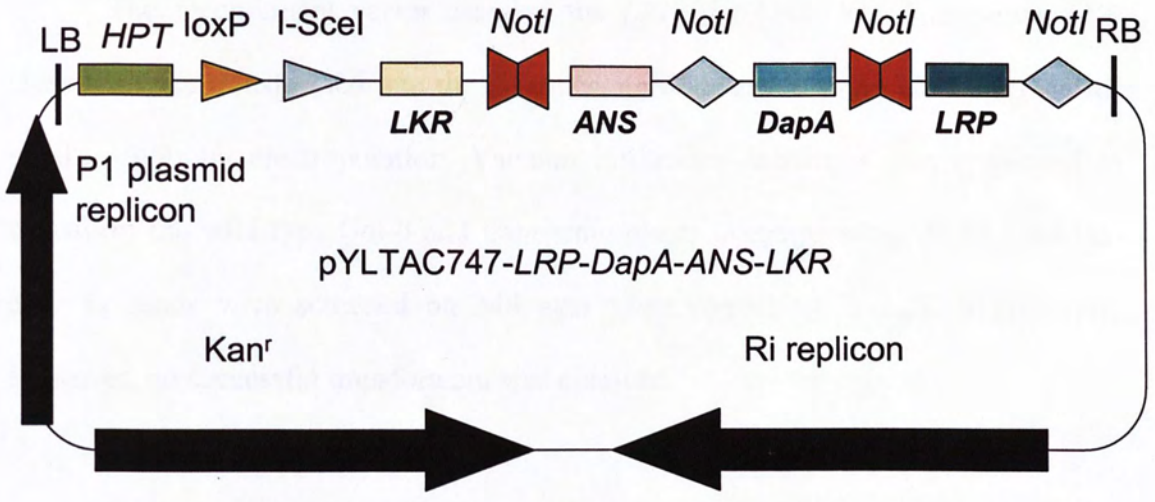


Figure 3.9 The schematic diagram of the acceptor vector pYLTAC 747H carrying the *LRP*, *dapA*, *ansB* and antisense *LKR* cassettes.

3.3.2 Transformation of *A. thaliana* with multigene vector

The recombinant vector carrying the *LRP*, *dapA*, *ansB* and antisense *LKR* cassettes were transformed into the disarmed *Agrobacterium* strain GV3101 (Merritt et al., 1999) by electroporation. Vacuum infiltration technique was employed to transform the wild type Col-0 and transgenic plants overexpressing *ASN1* (362-D2-d6). T_1 seeds were screened on MS agar plate containing 20mg/L hygromycin. However, no successful transformant was obtained.

Chapter 4 Discussion

Plant protein is a major source of dietary protein for human and livestock. However, most crop seeds, which are the major consumable part, are nutritionally incomplete due to insufficient synthesis and accumulation of several essential amino acids, such as Lys and Met (Galili and Hofgen, 2002). Manipulation of sink-source relationship to improve quantity and quality of seed proteins in crop seeds provides a cost-effective way to supply such amino acids. Metabolic pathways of aspartate family amino acids, which are all essential amino acids except asparagine, are major targets for seed quality improvement. In this study, we attempted to manipulate the N sink-source relationship by i) manipulation of aspartate family amino acid biosynthesis, and ii) manipulation of global regulation of nitrogen metabolism.

4.1 Characterization of transgenic plants with altered sink-source relationship of aspartate family amino acid metabolism

Aspartate family essential amino acids, especially lysine and methionine, are major targets for seed protein improvement and many strategies have been attempted to manipulate the content of aspartate family essential amino acids. These strategies can be categorized into three groups 1) increasing the supply of nitrogen resources from source to sink to provide an enhanced source for aspartate family amino acid biosynthesis; 2) deregulating the metabolic pathway to enhance the synthesis or reduce the catabolism of essential amino acids; 3) entrapping the free essential amino acids into appropriate seed storage proteins. There were also attempts to generate transgenic plants with a combination of two strategies, e.g. transgenic narbon bean expressing feedback insensitive AK and Brazil nut 2S albumin (Demidov *et al.*, 2003).

In this study, to test different strategies to enhance aspartate family amino acid, five transgenic lines of *A. thaliana* with altered sink-source relationship of aspartate family amino acids were employed (as mentioned in Section 1.7).

4.1.1 Total content of aspartate family amino acids remains steady in transgenic lines

The four aspartate family amino acids, Lys, Met, Thr and Ile, are derived from the common precursor aspartate. The data of amino acid analysis suggested that both the absolute and relative levels of the total content of aspartate family essential amino acids in mature seeds remained more or less unchanged in the five transgenic lines, compared to the wild-type Col-0.

For 35S-*ASN1* lines, the absolute and relative levels of Asx, Asp and Asn, in seeds increase significantly. Although there is an increased supply for the Asp (Asn can be converted to Asp by asparaginase), there is no significant increase in the total content of aspartate family essential amino acids. This is not surprising because the level of aspartate family amino acids is tightly regulated (Figure 1.2).

The other four lines are transgenic plants expressing an effective sink of Met or Lys (C-6-3 and 4-1-7), transgenic plants with deregulated feedback inhibition of AK-HSD (6-6-1) and transgenic plants with a combination of an effective sink of Lys and deregulated feedback inhibition of DHPS (8-1-1). The total content of aspartate family essential amino acids in seeds of these four lines remained unchanged. The accumulation of aspartate family essential amino acids of these transgenic plants may be due to the limited supply of the common precursor Asp.

4.1.2 Methionine content increases in phas-*PN2S* and phas-*MetL* transgenic plants

Met plays essential roles in cellular metabolism. Besides being a protein constituent, it is also the component of methionyl tRNA and plays a major role in initiation of translation (Hesse and Hoefgen, 2003). Its derivatives SAM serves as a primary methyl-group donor and also the precursor for metabolites such as ethylene, polyamines and vitamin B1 (Hesse and Hoefgen, 2003). Due to its biological importance and its deficiency in major crops, Met is one of the major targets of molecular manipulation.

Previous attempts to manipulate the Met content were mainly by expressing Met-rich proteins, and by engineering Met biosynthesis pathways. Seed-specific expression of Met-rich proteins, such as Brazil nut 2S albumin (BN2S) (Hesse *et al.*, 2001) and Sunflower seed 2S albumin (SSA) (Molvig *et al.*, 1997), successfully enhanced the Met content in seeds. However, due to the allergenic natures of BN2S and SSA, commercialization of transgenic crops expressing BN2S and SSA was hampered. For engineering of Met biosynthesis, a slight increase in the free Met content of seeds was reported in transgenic lines expressing feedback-insensitive AK in a seed-specific manner (Karchi *et al.*, 1993).

In this study, transgenic plants generated by both strategies were characterized to investigate and compare their performance on Met enhancement. Line C-6-3 is a transgenic line expressing Met-rich protein PN2S in seeds, while line 6-6-1 is a transgenic line engineering Met synthesis by expressing feedback-insensitive AK-HSD in seeds. In C-6-3, there was a significant increase in both the absolute and relative amounts of Met in seeds, compared to Col-0. In 6-6-1, there was also a small

but significant increase in the relative level of Met, while the increase in absolute level of Met was not statistically significant. This indicates that providing an effective sink by Met-rich protein is more efficient in enhancement of seed Met content than deregulation of AK-HSD. The increase in Met in C-6-3 was accompanied by a decrease in Lys content, but there was no change in Thr content. This suggested that when expressing Met-rich protein, Met synthesis will compete with Lys synthesis for aspartate semialdehyde, while the balance of Thr synthesis and Met synthesis will not be affected. This result is consistent with previous study that the increase in Met in transgenic narbon bean expressing BN2S in seed was associated with a reduction in free Lys, although the total Lys remained unchanged (Demidov *et al.*, 2003).

4.1.3 Relative lysine content increases in phas-*dapA*/phas-*LRP* transgenic plants

As cereal grains, which provide the major source of dietary protein for human and livestock, are limiting in essential amino acid Lys, Lys is considered as one of the major targets for seed protein improvement.

Three main strategies to improve the seed lysine content were 1) engineering of Lys synthesis pathway, 2) reduction of lysine catabolism, and 3) expressing Lys-rich proteins. Recently, there were attempts to manipulate Lys content by combining the strategies of engineering of Lys synthesis and reduction of Lys catabolism, and this resulted in a boost in Lys content in seeds (Zhu and Galili, 2003, 2004).

In this study, the transgenic expressing Lys-rich protein (4-1-7) and transgenic plants with a combination of engineering of Lys synthesis (by feedback insensitive DHPS) and expression of Lys-rich protein (8-1-1) were characterized to compare their

performance on Lys manipulation. From previous study of transgenic plants expressing LRP, despite that LRP accumulated and accounted for 3-10% of total seed soluble proteins, no increase in seed Lys content was observed (Cheng, 1999). The result of line 4-1-7 was consistent with the previous study. In line 8-1-1, there was a slight increase in relative Lys content, accompanied with a slight drop in Met content. The possible reason is that accumulation of Lys-containing proteins is limited by the level of free Lys, while deregulation of DHPS will channel more aspartate semialdehyde to Lys synthesis and supply free Lys for accumulation of Lys-containing proteins. This redirection of metabolic pathway reduces the precursor for Met synthesis and lowers the Met level.

While Lys synthesis seems to compete with Met synthesis in this study, in transgenic plant with deregulated DHPS and reduced Lys catabolism, the increase in Lys content was associated with a metabolically unanticipated increase in Met content (Zhu and Galili, 2004). As transgenic plants expressing deregulated DHPS also showed similar increase in Met content, the increase in Met should be attributed to the deregulation of DHPS but not the reduction of Lys catabolism. It was proposed that the increase in Met content is due to a plausible competition between Thr and Met biosynthesis, and reduction in Met catabolism (Zhu and Galili, 2004). However, in this study, the Met content in line 8-1-1 did not increase, but decreased slightly. One possible explanation is that the Met content was regulated by the level of free Lys. In line 8-1-1, there is an effective sink for free Lys and the level of free Lys should be lower than that of transgenic plant expressing deregulated DHPS only. To verify this, free amino acid level analysis could be performed to test the free Lys level in line 8-1-1. The finding that the Met content is positively correlated with Lys content (Zhu and

Galili, 2004) agrees with this explanation. However, the mechanism of how the Met level is regulated by the Lys level is unknown. One possibility is that Lys or its derivatives act as a signaling molecule to regulate the Met metabolism. The important roles of Lys in control of growth and development have been demonstrated by the negative physiological effects of free Lys accumulation in transgenic plants (Shaul and Galili, 1992; Ben-Tzvi Tzchori *et al.*, 1996; Lee *et al.*, 2001; Zhu and Galili, 2003). The fact that Lys content is tightly regulated by both synthesis and catabolism (Zhu and Galili, 2004) may probably because Lys is not only a protein constituent but also a signaling molecule (Anzala *et al.*, 2006).

4.1.4 Coordinated regulation of gene expressions of *akthr1* and *akthr2* with *GCN2* expression in transgenic plants with altered sink-source relationship

AK catalyzes the first committed step of the biosynthesis of aspartate family essential amino acids. There are two forms of AK, the threnonine sensitive bifunctional AK-HSD and the lysine sensitive monofunctional AK. Two genes, *akthr1* (Ghislain *et al.*, 1994) and *akthr2* (Rognes *et al.*, 2003), encoding AK-HSD were identified in *A. thaliana*. The two genes are subjected to spatial and temporal transcriptional control (Zhu-Shimoni *et al.*, 1997; Rognes *et al.*, 2003). For *akthr1*, northern blot analysis and expression of chimeric *akthr1* promoter-GUS construct suggested that *akthr1* is under transcriptional regulation by photosynthesis-elated signals but not by nitrogenous compounds (Zhu-Shimoni and Galili, 1998).

However, in this study, a down-regulation of *akthr1* expression was observed in transgenic plants with altered N sink-source relationship. Real-time PCR results showed that *akthr1* expression was down-regulated in transgenic line C-6-3, 4-1-7, 6-

6-1, and 8-1-1 during silique development. A similar down-regulation was also found for *akthr2*. This down-regulation of genes encoding AK-HSD enzyme may be a obstacle limiting the enhancement of aspartate family amino acids in these lines. GCN4-like sequences were identified in promoters of *akthr1* and *akthr2*, while opaque2-like sequence was identified in *akthr1* (Rognes *et al.*, 2003). Interestingly, a down-regulation of *GCN2* expression was also observed in those lines with down-regulation of *akthr* genes, suggesting a correlation between GCN2 expression and expression of *akthr1* and *akthr2*. However, the reason why *GCN2* expression is down-regulated in these lines is unknown and free amino acid analysis of developing siliques may give clues to this question.

4.2 GCN system in plants

Under amino acid starvation, multiple amino acid biosynthetic pathways will be activated to synthesize more amino acids to cope with starvation. The GCN system is responsible for this kind of GAAC and was first identified in yeast (Wek *et al.*, 1989; Hinnebusch, 1994; Hinnebusch, 1997, 2005). The cross-regulation of amino acid biosynthesis (Guyer *et al.*, 1995; Zhao *et al.*, 1998; Noutoshi *et al.*, 2005) and existence of homologs of GCN components (Chang *et al.*, 1999; Chang *et al.*, 2000; Gil *et al.*, 2000; Zhang *et al.*, 2003) suggested the existence of a GCN system in plants.

4.2.1 Transcriptional regulation of GCN2 in *A. thaliana*

In yeast, GCN2 is activated by the binding of uncharged t-RNA and facilitated by interactions with GCN1 and GCN20 (Zhu *et al.*, 1996; Marton *et al.*, 1997; Garcia-Barrio *et al.*, 2000). The regulation of yeast GCN2 is under post-translational control

and the GCN2 mRNA and protein levels did not response to amino acid starvation (Wek *et al.*, 1990). However, the expression of *GCN2* increases in both wild-type Col-0 and 35S-GCN2 upon amino acid starvation (generated by azaserine treatment). This suggests that in *A. thaliana*, GCN2 is subjected to transcriptional control. The down-regulation of *GCN2* in developing siliques of transgenic plants with altered N sink-source relationship is another evidence for the transcriptional control of *GCN2*. It is not surprising that plant GCN2 and yeast GCN2 are subjected to different regulatory mechanism because another N regulatory system, the PII system, is also subjected to different regulatory mechanism in different organisms. The signal transduction protein PII is subjected to post-translational regulation by uridylylation in *E. coli* (Atkinson *et al.*, 1994), and by phosphorylation in cyanobacteria (Lee *et al.*, 2000). In plants, the expression of *GLB1*, the gene encoding PII, is regulated transcriptionally by light as well as carbon and nitrogen metabolites (Hsieh *et al.*, 1998).

4.2.2 Regulation of amino acid biosynthesis by GCN system

4.2.2.1 Regulation of *akthr1* and *akthr2* by GCN2

GCN2 homolog was identified in *A. thaliana* by yeast functional complementation (Zhang *et al.*, 2003). Although AtGCN2 can complement the yeast *gcn2* mutant, there is no direct evidence that GCN2 plays a role in regulation of amino acid biosynthesis *in planta*.

In addition to the coordinated down-regulation of *akthr* genes and *GCN2* in developing siliques of transgenic plants with altered N sink-source relationship, induction of *akthr1* and *akthr2* were observed in developing siliques of transgenic

plants overexpressing *GCN2*. The induction of *akthr1* and *akthr2* in seedlings of 35-*GCN2* transgenic plants indicates that GCN2 plays a role in both vegetative tissues and reproductive tissues. Treatment of azaserine, a glutamate analog, will cause a global deficiency in amino acid pools. This generated a condition of amino acid starvation, and the expression of *akthr* genes was induced by this starvation condition. On the other hand, overexpression of *GCN2* can mimic the condition of amino acid starvation and induce *akthr1* and *akthr2* gene expression even in non-starved condition. These findings further strength the notion that *GCN2* regulates the gene expression *akthr1* and *akthr2*. As mentioned in Section 4.1.4, GCN4-like sequences were identified in promoters of *akthr1* and *akthr2*, while opaque2-like sequence was identified in *akthr1* (Rognes *et al.*, 2003). Moreover, the GCN4-like sequence in *akthr1* promoter plays a functional role in expression of *akthr1* (Zhu-Shimoni and Galili, 1998). These findings agree with our notion that *akthr1* and *akthr2* are the targets of the GCN system in *A. thaliana*. A possible model is that the amino acid starvation will induce the expression of *GCN2* and GCN2 will trigger the downstream pathway to induce the transcriptional of amino acid biosynthetic gene *akthr1* and *akthr2* by acting on the GCN4-like *cis*-element.

4.2.2.2 GCN4 homolog in plants?

In addition to GCN2, homologs of GCN1 and GCN20 were also identified in *A. thaliana* (Kato *et al.*, 2004), and homolog of eIF2α were identified in wheat (Chang *et al.*, 1999; Chang *et al.*, 2000). However, the homolog of a key GCN component, GCN4, is still missing in plants. The bZIP transcription activator opaque2 can functional complement yeast *gcn4* mutant under amino acid starvation (Mauri *et al.*, 1993), suggesting that opaque2 maybe a functional homolog of GCN4. The

opaque2 maize mutant exhibits a high seed Lys content and a reduction in LKR transcription and activity (Kemper *et al.*, 1999). Opaque2 was also found to regulate the biosynthesis of Lys via *ask1* in maize *opaque2/ask1* mutant (Brennecke *et al.*, 1996). The regulation of amino acid metabolism by opaque2 suggests that opaque2 play a role in transcriptional activation of amino acid biosynthetic genes as that of GCN4 in yeast. Proteomic studies of the GCN2 transgenic plants, such as 2D-PAGE, and interaction with GCN4-like and opaque2-like sequences may help to screen for possible candidates of GCN4 homologs in plants.

4.2.2.3 Regulation of amino acid metabolism by GCN system

Beside *akthr* genes, there are also other genes containing GCN4-like or opaque2-like sequences in their promoters. GCN4-like motif have been found in promoters of many plants genes involved in N metabolism and storage protein genes, and the GCN4-like motif of a barley storage protein gene was found to response to nitrogen (Muller and Knudsen, 1993). Opaque2 was found to regulate several genes with GCN4-like sequences in their promoter (Yunes *et al.*, 1994; Holdsworth *et al.*, 1995; Maddaloni *et al.*, 1996; Wu *et al.*, 1998). For instance, GCN4-like sequences found in promoter of a rice glutelin gene acts as a *cis*-regulatory element in regulation of seed-specific expression and was activated by opaque2 in transgenic rice (Wu *et al.*, 1998). LKR for Lys catabolism has a opaque2-like sequence in promoter and the LKR activity was found to regulated by opaque2 (Arruda *et al.*, 2000; Ferreira *et al.*, 2005). These genes with GCN4-like or opaque2-like sequence in the promoters are possible targets for the GCN system in plants. Studying the expressions of these candidate genes in GCN2 transgenic plants will give information whether the gene is subjected to the regulation of GCN system.

4.3 Generation of transgenic plants with a combination of altered sink-source relationship

Gene cassettes of asparaginase, feedback insensitive dihydrodipicolinate synthase, lysine-rich protein and lysine-2-oxoglutarate reductase were first cloned into two donor vectors. These cassettes were subsequently introduced into a transformable acceptor vector by Cre-recombination. A transformable acceptor vector carrying four target genes was successfully constructed. Transformation of *Arabidopsis thaliana* overexpressing asparagine synthetase by *Agrobacterium*-mediated transformation has been carried out. The construct is designed to (i) increase the supply of nitrogen resources from source to sink, (ii) increase aspartate level in seeds, (iii) deregulate product feedback inhibition for lysine biosynthesis, (iv) reduce catabolism of lysine, and (v) entrap accumulated free lysine into lysine-rich seed proteins. Several rounds of transformation were carried out; however, no transformant was obtained. This may due to 1) the huge size of the plasmid that lowers the transformation efficiency; 2) accumulation of free lysine which is toxic to the plants; 3) undesirable mutation or recombination of the plasmid making it untransformable.

As mentioned in Section 1.5.3, another system for transformation of multiple genes simultaneously by employing the rare-cutting homing endonuclease was developed (Goderis *et al.*, 2002). This system can be employed to generate transgenic plants with a combination of altered sink-source relationship. The gene cassettes of asparaginase, feedback insensitive dihydrodipicolinate synthase, lysine-rich protein and lysine-2-oxoglutarate reductase can be released from the two donor vectors and

then cloned into auxiliary vectors, and then transferred to the binary vector by the use of appropriate homing endonucleases.

Chapter 5. Conclusion and Future Prospective

Five different transgenic plants with altered N sink-source relationship were characterized and compared to the wild-type Col-0. They are transgenic plants with an enhanced source of Asn transported from source tissues (D2d6), transgenic plants expressing an effective sink for Met or Lys (C-6-3 and 4-1-7 respectively), transgenic plants with deregulated feedback inhibition of AK-HSD (6-6-1) and transgenic plants with a combination of an effective sink of Lys and deregulated feedback inhibition of DHPS (8-1-1). These manipulations cannot improve the total content of Asp family amino acids in the seeds. The down-regulation of *akthr1* and *akthr2*, the two genes encoding AK-HSD which catalyzes the first committed step of Asp family pathway, maybe one of the obstacles for improving the total content of Asp family amino acids in these lines.

While expressing an effective sink for Met can effectively enhance the Met content (in C-6-3), expressing an effective sink for Lys cannot effectively improve the Lys content (in 4-1-7), even when combined with deregulated Lys synthesis (in 8-1-1). The Lys content is regulated more tightly than Met and cannot be enhanced effectively without reducing Lys catabolism.

The coordinated down regulation of GCN2 with the GCN4-like motifs-containing *akthr1* and *akthr2* suggests that GCN2 regulates the expressions of *akthr1* and *akthr2*. The up-regulation of *akthr1* and *akthr2* by *GCN2* overexpression further strengthens this notion. These results suggest that a functional GCN system does exist in plants and suggest a correlation of the GCN system with amino acid biosynthesis during seed development.

In this study, only one transgenic line overexpressing *GCN2* were employed. More *GCN2* transgenic lines should be screened for future study to confirm the results of this research. To gain more understanding of GCN system in plants, free amino acid levels of the developing siliques of the transgenic plants with down-regulated *GCN2* expression should be studied to investigate how *GCN2* expression is regulated. Moreover, the free and total amino acid content in seeds of transgenic plants overexpressing *GCN2* should be studied to explore the feasibility of seed protein improvement by combining the manipulation of *GCN2* and manipulation of N sink-source relationship.

In this study, the generation of transgenic plants with a combination of manipulation of sink-source relationship of aspartate family amino acids is not successful. With the gene cassettes cloned, another system for transformation of multiple genes simultaneously by employing the rare-cutting homing endonuclease (Goderis *et al.*, 2002) can be employed to generate the transgenic plants in future study.

Appendix I: The major chemicals and reagents used in this research

1. Ammonium acetate	Ajax 27
2. Ampicillin	Sigma A9518
3. Agarose	GibcoBRL 15510-027
4. Bacto-peptone	Difco 0118-01-8
5. Bacto™ Agar	Difco 214010
6. Bromophenol blue	Merck 8122
7. Cetyldimethylethylammonium bromide (CTAB)	Sigma C5335
8. Chloramphenicol	Amresco E868
9. Chloroform	Merck 3445
10. dNTP	Boehringer 1277049
11. EDTA, disodium salt	Sigma E5143
12. Ethanol (absolute)	Merck 100986
13. Ethidium bromide	Sigma E7637
14. Gentamicin sulfate	Sigma G3632
15. Glacial acetic acid	Sigma A4508
16. Hydrochloric acid (36%)	Ajax 1364
17. Iso-amylalcohol	Merck 100979
18. Isopropanol	Labscan C2519
19. Isopropyl b-D-thiogalactopyanside (IPTG)	Boehringer 1411446
20. Kanamycin, monosulfate	Sigma K4000
21. Magnesium chloride	Sigma M9272
22. Magnesium sulphate	Ajax 302
23. β-mercaptoethanol	Sigma M6250

24. MES	Sigma 3023
25. Metro-mix soil	Hummert 10-0325
26. Murashige & Skoog salt mixture	GibcoBRL 11117-017
27. Phenol	M&B
28. Silwet-77	Lehle seeds
29. Sodium acetate, anhydrous	Sigma S2889
30. Sodium chloride	RDH 31434
31. Sodium dodecyl sulfate	B/M 1028693
32. Sodium hydroxide	Merck 6498
33. Sucrose	Sigma S1888
34. Tris/ HCl	Amresco 0826

Appendix II: Major buffers, solutions and mediums used in this research

ATP (10 mM)

Appropriate amount of solid ATP is dissolved into 25mM Tris-Cl (pH 8.0). The resultant solution could be stored as aliquots at -20^0C.

Bromophenol blue solution (0.25%, w/v)

2.5mg of solid bromophenol blue is dissolved into 1ml of sterile water. The solution could be stored in room temperature.

Bromophenol blue loading dye (10x)

0.25% bromophenol blue in 30% glycerol

B5 vitamin (1000X)

1000mg myo-inositol, 100mg thiamine-HCL, 10mg nicotine acid, 10mg pyridoxine-HCl. Fill up to 10 ml with water.

Calcium chloride (2.5M)

11g of $CaCl_2 \bullet 6H_2O$ is dissolved into final volume of 20ml distilled water. The resultant solution is sterilized by passing through a 0.22 μm filter and could be stored in 1ml aliquots at 4^0C.

CTAB extraction buffer

0.1M Tris-HCl (pH8), 1.4M NaCl, 0.1M EDTA (pH8), 2% (w/v) CTAB, 1% (w/v) Polyvenylprolidone and 0.2 % freshly added β-mercaptoethanol.

Deoxyribonucleoside triphosphate (dNTPs)

Each dNTP is dissolved in distilled water to concentration of 100mM. The pH of each

solutions are adjusted to 7.0 using 0.05M Tris base. The solution is then diluted with distilled water to a final concentration of 50mM dNTP. Each is store separately at -70^0C in small aliquots.

DEPC-treated water

1ml of DEPC was mixed with 1 liter distilled water and kept overnight at room temperature before autoclaving.

EDTA (0.5M, pH 8.0)

186.1 g of disodium EDTA•$2H_2O$ is dissolved to 800ml of distilled water. The pH of the mixture is adjusted to 8.0 with ~20g of NaOH pellet. The resultant solution is dispensed into aliquots and sterilized by autoclaving.

Ethidium bromide (10mg/ml)

1g of ethidium bromide is added to 100ml of distilled water. The mixture is stirred on magnetic stirrer for several hours until all dye has dissolved. The solution could be stored in either container wrapped with aluminum foil or dark bottle in room temperature.

Germination medium (MS medium)

2.15g/L MS salt, 1%sucrose, 0.05%MES and 0.9% Batoagar

Glycerol (10% v/v)

1 volume of molecular-biology-grade glycerol is diluted into 9 volume of sterile pure H_2O. The solution is sterilized by passing through a prerinsed 0.22 μm filter and could be stored in 200ml aliquots.

Infiltration medium

2.2g MS salts, 1x B5 vitamins, 50g sucrose, 0.5g MES and 200ul Silwet

IPTG (20% w/v, 0.8M)

2g of IPTG us dissolved into 8ml of distilled water. The volume of solution is added up to 10 ml and then sterilized by passing through a 0.22 μm disposable filter. The solution could then be stored as 1 ml aliquots at -20^0C.

LB agar plate

25g/L LB powder and 15g/L bacto-agar, autoclave

LB broth

25g/L LB powder, autoclave

NaOH (10M)

400 g of NaOH pellets are slowly added to 800ml of water in a plastic beaker placed in ice bath with stirring. The volume of mixture is adjusted to 1 L with distilled water. The solution could be stored in a plastic container at room temperature.

NaCl (5M)

292 g of NaCl is dissolved in 800ml of distilled water and the volume of solution is adjusted to 1 L by adding more distilled water. The solution is stored as aliquots in room temperature.

0.1M Potassium phosphate buffer at pH 7.0

61.5 ml 1M K_2HPO_4 is mixed with 38.5 ml 1M KH_2PO_4. The combined solution is then diluted to 1 L with distilled water.

RNA extraction buffer

0.1 M Tris-HCl, pH 8.0, 0.1 M LiCl, 0.1 M EDTA, and 1% SDS

SDS (20% w/v)

200 g of electrophoresis grade SDS is dissolved in 900 ml of distilled water. The mixture

is heated to 68'C and stirred with a magnetic stirrer. The pH of the solution is adjusted to 7.2 with concentrated HCl and the volume of solution is added up to 1 L with distilled water. The solution could be stored in room temperature.

Sodium acetate (3M, pH 5.2 and pH 7.0)

408.3 g of sodium acetate•$3H_2O$ is dissolved into 800 ml of H_2O. The pH of the solution is adjusted to 5.2 with glacial acetic acid or adjusted to 7.0 with diluted acetic acid. The volume of solution is added up to 1L with distilled water. The solution could then be dispensed into aliquots and sterilized by autoclaving.

SOC medium

20g of tryptone, 5g of yeast extract and 0.5 g of NaCl is dissolved into 950 ml of distilled water. 10 ml of 250mM solution of KCl is added into the mixture and the pH of the medium is adjusted to 7.0 with ~0.2ml 5M NaOH. The volume of the solution is added up to 1 L with distilled water. The solution is sterilized by autoclaving and then allowed to cool down to 60^0C or less. 20 ml of sterile 1 M glucose solution is added. Just before use, 5ml of sterile 2M $MgCl_2$ is added into mixture.

50X TAE

242g of Tris base and 57.1ml of glacial acetic acid are added into 100ml 0.5M EDTA at pH8.0. The mixture is then added up to 1L with distilled water.

1M Tris-Cl at pH 8.0

121.1g of Tris base are dissolved into 800ml of distilled water. The solution is allowed to cool down to room temperature and the pH of the solution is then adjusted to 8.0 by adding nearly 42 ml conc. HCl. The solution is diluted to 1L with distilled water. The resultant solution is then dispensed into aliquots and then sterilized by autoclaving.
(Note: 1. The pH of Tris is greatly affected by temperature, i.e. decrease 0.03 pH unit for each 1^0C decrease; 2. Yellow color of 1M solution indicates its poor quality of stock).

10X Tris EDTA (TE) at pH 8.0

10mM EDTA(pH 8.0) are mixed with 100mM Tris-Cl (pH8.0). The solution is sterilized by autoclaving for 20 minutes. The solution could be stored in room temperature.

X – gal solution (2% w/v)

X – gal is dissolved into dimethylformamide in glass or polypropylene tube so that the its final concentration is 20mg/ml. The tube containing the solution is wrapped with aluminum foil and stored at -20^0C.

YEP medium

10g tryptone, 10g yeast extract and 5g NaCl. Fill up with 1 litre with water.

Appendix III: Commercial kits used in this research

1. ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer 402078)

2. Prep-A-Gene® purification systems (Bio-Rad 732 6011)

3. Wizard™ Plus Minipreps DNA purification systems (Promega A7510)

Appendix IV: Major equipment and facilities used in this research

Equipment/facilities	Company and catalogue number
Real time PCR system	ABI Prism 7700
Biological safety cabinet	Baker SG600E 59419
Centrifuge	Eppendrof 5415C
Centrifuge J2-MI	Bechman T373 with JA-14 rotor
Environmentally-controlled growth chamber for soil grown plants	South China House of Technology (Development) Limit
Gel 1000UV Fluorescent Gel Doc	Bio-Rad 200015450
Gene Pulser	Bio-Rad 165-2076
Genetic Analyzer ABI Prism 310	Perkin elmer 96030481
Microcooler II	Bockel Scientific 260010
Orbital shaker	Lab line 4628-1
Power supply MIDI MP-250	Life technologies 4801311
Programmable Thermal cycle	MJ research PTC-100 96VHB 200003879
Refrigerated Centrifuge	Eppendorf 5810R
Speedvac apparatus	Labconco 79840-01
TELCO incubator	Cole-Parmer 39352-02
Ultrapure water	Millipore PROG00001

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